Salivary Gland Tumors with Myoepithelial Differentiation

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2008

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Salivary Gland Tumors with Myoepithelial Differentiation:

Immunoprofiling and Genomic Analysis

The studies described in this thesis were carried out at the departments of Oral and Maxillofacial Surgery/Oral Pathology at the VU University Medical Center and Academic Centre for Dentistry, Amsterdam, the Netherlands (head: Prof.dr. I. van der Waal). This work was supported financially by the Interuniversity Research School (IOT), Amsterdam, the Netherlands.

ISBN: 978-90-9023616-2

Printed by PrintPartners Ipskamp B.V., Enschede, the Netherlands (2008)
Cover design by Christianne Renard
Layout by Hedvig Vékony

VRIJE UNIVERSITEIT

SALIVARY GLAND TUMORS WITH MYOEPITHELIAL DIFFERENTIATION: IMMUNOPROFILING AND GENOMIC ANALYSIS

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
prof.dr. L.M. Bouter,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Tandheelkunde
op woensdag 10 december 2008 om 10.45 uur
in de aula van de universiteit,
De Boelelaan 1105

door

Hedvig Vékony

geboren te Kecskemét, Hongarije

promotoren: prof.dr. E. Bloemena
prof.dr. I. van der Waal

Contents

List of abbreviations

ACC	Adenoid cystic carcinoma
Array CGH	Microarray-based Comparative Genomic Hybridization
BME	Benign myoepithelioma
EGFR	Epidermal growth factor receptor
FISH	Fluorescence in situ hybridization
FGF	Fibroblastic growth factor
FGFR	Fibroblastic growth factor receptor
FUP	Follow-up
LOH	Loss of heterozygosity
PA	Pleiomorphic adenoma
PcG	Polycomb group
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
pRb	Retinoblastoma protein
PRC	Polycomb repressive complex
SMA	Smooth muscle actin
WECCA	Weighted clustering of called aCGH data

CHAPTER 1

General Introduction

Chapter 1

1 Salivary Glands

The human salivary gland system can be divided into two distinct exocrine groups. The paired **major salivary glands** are represented by the parotid, the submandibular, and the sublingual glands. The parotid glands are located along the posterior surface of the mandible and are the largest salivary glands. The submandibular glands are located beneath the floor of the mouth and the sublingual glands are located underneath the tongue (Figure 1). The numerous **minor salivary glands** are widely scattered throughout the upper aerodigestive submucosa (i.e. palate, lips, oropharynx, larynx, parapharyngeal space).[1] Heterotopic minor salivary glands can also occur at unexpected sites, including lymph nodes, the capsule of the thyroid gland, facial bones, and the hypophysis.[2]

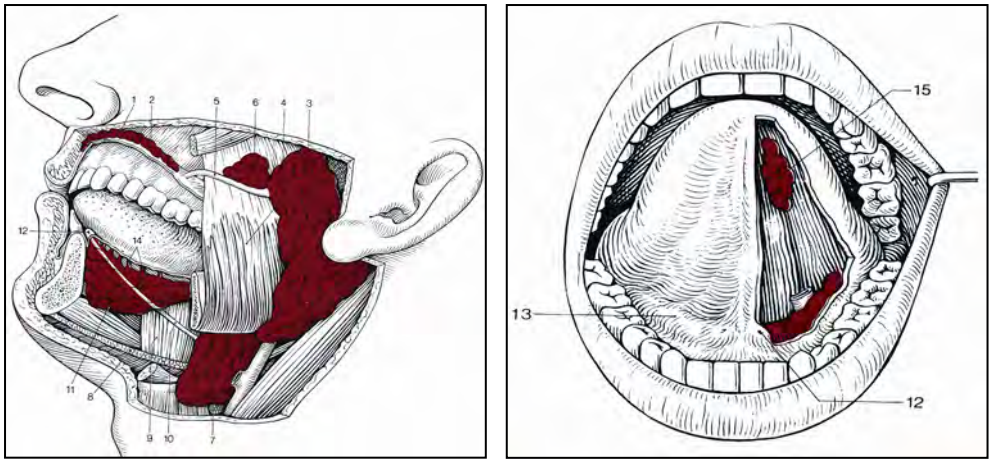

Figure 1 Anatomic location of the major and minor salivary glands in combination with their associated draining duct structures. The parotid (3), the submandibular (7), and the sublingual (11, 14 and 15) glands are depicted together with numerous minor salivary glands (1, 2, 6). The muscularis masseter (4), mylohyoideus (8), hyoglossus (9) and the ducti parotideus (5) and submandibularis (10) are shown together with the caruncula sublingualis (12) and plica sublingualis (13). (Source: Sesam Atlas of Anatomy: Internal Organs. 12th Ed, Bosch en Keuning NV, Baarn)

Salivary gland development is an example of branching morphogenesis, a process fundamental to many developing organs, including lung, mammary glands, pancreas, and kidney.[3] During the 6th-8th embryonic week, the major salivary glands develop as outpouchings of oral ectoderm and grow inwardly into the surrounding mesenchyme.[4] The parotid gland is the first to appear and is divided by the facial nerve development by about the 10th week into superficial and deep portions.[5] Late encapsulation of the gland is responsible for the entrapment of lymphatics in its parenchyma. Salivary epithelial cells are often included within these lymph nodes and are probably participating in the development of Warthin's tumors.[6] Minor salivary glands arise from oral ectoderm and nasopharyngeal endoderm and their morphogenesis starts after the major salivary glands. For overall glandular growth, nervous system involvement and tightly regulated signaling pathways (sonic hedgehog (Shh) and the fibroblastic growth factor (FGF) family of receptors) are crucial.[7,8]

1.1 Anatomy

The basic secretory units of the salivary glands consist of the spherical **acinus**, the **myoepithelial cell**, the **intercalated duct**, the **striated duct**, and the **excretory duct** (Figure 2). The primary secretion is produced in the acini and the duct system carries the saliva to the oral cavity. Myoepithelial cells embrace the secretory acini and the intercalated ducts, while the striated ducts and the subsequent conducting portion are supported by basal cells. The secretory units are divided into three types: (1) **serous** (thin, watery, proteinaceous, amylase secreted), (2) **mucous** (viscous, glycoprotein-rich, sialomucins secreted) and (3) **mixed**.[2]

The myoepithelial cells (or 'basket cells') are located between the basement membrane and the basal plasma membrane of the acinar cells. Besides the salivary glands, these cells are also found in breast, sweat, and lachrymal glands.[9,10] Myoepithelial cells are flat, variable however in morphology, and cannot be reliably identified by light microscopy. They possess dendritic processes that extend over the epithelial surface and embrace the secretory acini. The most profound characteristic of these cells is their dual basal epithelial-myoid nature.[9] In addition to basal cytokeratin expression (i.e. cytokeratin 5 and 14),

myoepithelial cells also contain cytoplasmic myofilaments, including actin, tropomyosin, and myosin.[2,11-14] The smooth muscle features provide contractility to the myoepithelial cells, allowing them to increase the pressure on the secretory cells and to speed up the outflow of the saliva.[15,16]

Salivary glands are classified according to the predominance of the types of secretory units. The parotid gland is almost exclusively serous while the submandibular gland is mixed, although the serous component predominates (~90%). The sublingual gland is also mixed, but it is mainly mucous in type.[2]

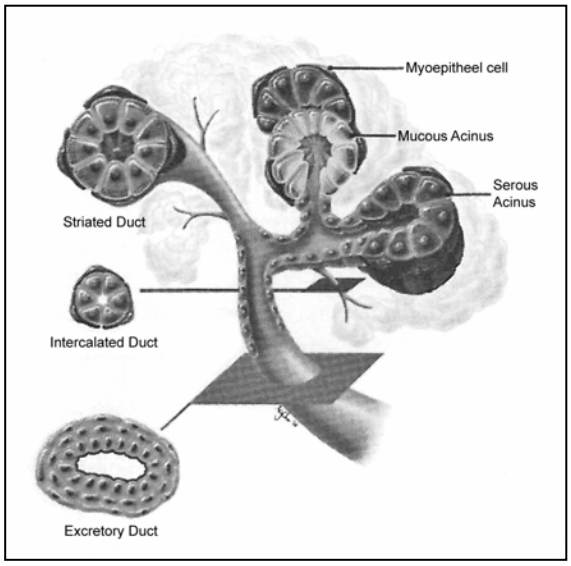

Figure 2 Overview of a terminal portion of the submandibular gland. One layer of myoepithelial cells invests the terminal secretory ducto-acinar unit, which consists of the intercalated duct and the acinus. (Source: Atlas of Clinical Oncology, Cancer of the Head and Neck. JP. Shah, editor, Chapter 2, p.25)

1.2 Saliva

The primary function of the salivary glands is the production and secretion of saliva. The autonomic nervous system controls both the volume (about 1000-1500 ml daily) and the type of saliva produced. Although it consists for approximately 99% of water, saliva is critical to preserve and maintain the health of oral tissues.[17] For instance, the secreted nonspecific antibacterial enzyme lysozyme serves as a main non-immune defense mechanism and controls bacterial colonization. Furthermore, carbonic acid-bicarbonate in saliva serves as a buffering system, maintaining the integrity of tooth enamel.

2 Salivary Gland Tumors

Tumors of the salivary glands display one of the greatest diversities of histology among human cancers. A broad morphologic spectrum exists between different tumor types and sometimes even within an individual tumor mass. In addition, the occurrence of hybrid tumors, dedifferentiation and the propensity of some benign salivary gland tumors to progress to malignancy make this group of lesions one of the most interesting and challenging in the head and neck.

2.1 Histogenesis and Classification

In the last decades, the numerous morphologic subtypes of salivary gland neoplasms have evoked considerable interest in their histogenetic origin. Determination of the cell type(s) involved in tumor induction in normal salivary gland tissue has led to the proposal of various theories. The first hypothesis was based on the positioning of proliferating cells in the salivary glands. Histologic observations in fetal salivary gland tissue suggested that the outer (basal) layer of cells give rise to the inner (luminal) layer. This **'semipluripotent bicellular reserve cell theory'** was proposed by Eversole in 1971 and refined by Batsakis and colleagues six years later.[18-20] In this concept, excretory basal cells only give rise to mucoepidermoid and squamous carcinomas, whereas the intercalated luminal cells are responsible for lesions like pleiomorphic and monomorphic adenomas, adenoid cystic carcinomas, and acinic cell carcinomas. The secretory acinar cells, being highly differentiated, are assumed to be incapable of cell division and thus play a minimal role in tumor formation. In 1989, Batsakis *et al.* evoked a **'pluripotent stem cell model'** to explain the phenotypic heterogeneneity seen within salivary gland neoplasms.[21] In this postulate, salivary gland tumors arise from a population of pluripotent stem cells present in the intercalated duct system where they are responsible both for the regeneration within the gland and for the development of metaplasia. Recent investigations however provided accumulating evidence that all mature cell types in salivary gland tissue are capable of proliferation,[22,23] giving rise to the **'multicellular concept'** of tumor histogenesis.[24] This latest concept

suggests that any of the cell types in salivary gland tissue is capable of giving rise to any of the various types of tumor in this organ.

Classification of salivary gland tumors is essentially based on morphology and histologic aspects of these tumors correlate with the normal salivary gland structure as described in section 1.1 (Figure 3). However, this histologic similarity does not necessarily imply that a particular tumor arises from the structure it mimics.[25]

The morphologic complexity of salivary gland tumors is further illustrated by the fact that in the latest revision of the World Health Organization (WHO) Histological Classification of Salivary Gland Tumors nearly forty different epithelial subtypes are recognized (Table 1).[1]

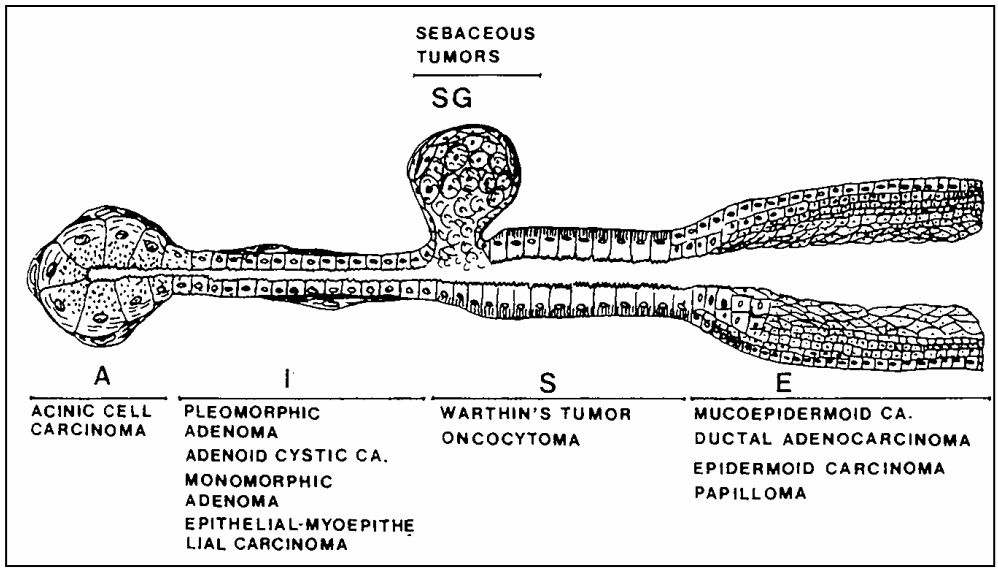

Figure 3 Morphologic similarity of different types of salivary gland tumors and the various epithelial structures of the salivary gland. Acinus (A), intercalated duct (I), striated duct (S), sebaceous gland (SG), and excretory ducts (E). (Source: Martinez-Madrigal, F *et al*. Am J Surg Pathol 1989)

2.2 Epidemiology

Salivary gland tumors are relatively uncommon neoplasms, representing 2%-6.5% of all head and neck neoplasms. Their global annual incidence varies from 0.4-13.5 cases per 100.000 people.[26,27] Most salivary gland tumors (~70%) originate in the parotid glands, 7-11% occur in the submandibular glands, less than 1% in the sublingual glands and 9%-23% in the minor glands. Although benign tumors account for 63% to 78% of all salivary gland neoplasms, the proportion of malignant tumors varies significantly by site and is the greatest in the sublingual glands, tongue, floor of the mouth, and retromolar area.[1,28]

The average ages of patients with benign and malignant tumors are 46 and 47 years, respectively and the peak incidence of most of the specific types is in the sixth and seventh decades. Among all patients, the most common tumor type is the pleiomorphic adenoma (PA), accounting for about 50% of all tumors. Warthin tumor is second in frequency among benign tumors and, in most large studies, mucoepidermoid carcinoma and adenoid cystic carcinoma are the most common malignant tumors.[1,29,30]

2.3 Etiology

Knowledge about the etiology of salivary gland tumors is still limited. However, there is compelling evidence that exposure to ionizing radiation increases the risk of salivary gland tumor development.[31,32] Increased occupational risks involve exposure to asbestos, nickel compounds or silica dust.[33,34] Furthermore, employment in the woodworking, rubber industries and beauty salons has also been implicated in a significantly higher incidence of salivary gland carcinomas.[35,36] Development of Warthin tumors showed a strong association with cigarette smoking.[37]

Malignant epithelial tumors	Benign epithelial tumors
Acinic cell carcinoma	Pleiomorphic adenoma
Mucoepidermoid carcinoma	Myoepithelioma
Adenoid cystic carcinoma	Basal cell adenoma
Polymorphous low-grade adenocarcinoma	Warthin tumor
Epithelial-myoepithelial carcinoma	Oncocytoma
Clear cell carcinoma, NOS	Canalicular adenoma
Basal cell adenocarcinoma	Lymphadenoma
Sebaceous carcinoma	Sebaceous lymphadenoma
Sebaceous lymphadenocarcinoma	Non-sebaceous lymphadenoma
Cystadenocarcinoma	Inverted ductal papilloma
Low-grade cribriform cystadenocarcinoma	Intraductal papilloma
Mucinous adenocarcinoma	Sialadenoma papilliferum
Oncocytic carcinoma	Cystadenoma
Salivary duct carcinoma	
Adenocarcinoma, NOS	
Myoepithelial carcinoma	
Carcinoma ex pleiomorphic adenoma	
Carcinosarcoma	
Metastasizing pleiomorphic adenoma	
Squamous cell carcinoma	
Small cell carcinoma	
Large cell carcinoma	
Lymphoepithelial carcinoma	
Sialoblastoma	

Table 1 The 2005 revision of the World Heath Organization Histological Classification of Epithelial Tumors of the Salivary Glands. NOS – not otherwise specified (Source: WHO Classification of Tumours: Head and Neck Tumours. IARC*Press*, Lyon, 2005)

2.4 Therapy and Prognosis

Treatment of salivary gland tumors is challenging because of their rarity and their unpredictable biologic behavior. Malignant neoplasms are marked by frequent locoregional failure and distant metastasis, often occurring years after diagnosis.[26,38]

Currently, treatment of choice is surgery and the goal, if possible, consists of total resection. Postoperative adjuvant radiation therapy is indicated for most patients with malignant tumors, but it only seems to improve locoregional control and not patient survival.[39,40] Even in case of radical excision, most salivary gland tumors are extremely difficult to treat. In adenoid cystic carcinoma for instance, distant metastases frequently develop even when locoregional control has been achieved.[41] It is assumed that micrometastases develop in the very early stages of the tumor, which can only be treated with additional chemotherapeutic agents. Detection of high levels of tyrosine kinases (e.g. c-kit, EGFR) in adenoid cystic carcinoma has initiated the application of systemic chemotherapy (e.g. Imatinib mesylate, Lapatinib) in recent years, but results still remain unsatisfactory.[42-46]

Prognosis and survival statistics following treatment of malignant tumors have been difficult to interpret, since the clinical course of salivary gland tumors is generally insidious and a long-term follow-up is required to determine the outcome. Factors usually considered to greatly influence prognosis are grade, tumor type, locoregional failure, presence of node metastases, and clinical stage.[39] All these factors emphasize the importance of early diagnosis of salivary gland tumors.

3 Salivary Gland Tumors with Myoepithelial Differentiation

Over the past decade, investigations on the natural tumor suppressing nature of myoepithelial cells have gained much scientific interest.[10,47,48] Several i*n vitro* and *in vivo* experiments, especially from studies of Sternlicht *et al.,* revealed that myoepithelial cells secrete relatively low levels of matrix-degrading proteinases and relatively high levels of maspin and several other anti-invasive proteinase inhibitors.[49,50] This

causes the accumulation rather than the degradation of extracellular matrix material. Furthermore, myoepithelial cells have been shown to induce epithelial morphogenesis and differentiation, and to inhibit tumor cell invasion and angiogenesis.[51]

These inherent anti-tumor properties are likely to contribute to their tendency to resist neoplastic transformation. Generally, myoepithelial tumors are uncommon, have a low-grade nature, and a vast majority of them is benign.[52] In the rare malignant cases however, they display aggressive biologic behavior and a poor survival. Three different salivary gland tumors with myoepithelial differentiation, which are the focus of this thesis, are discussed in the next subchapters.

3.1 Adenoid Cystic Carcinoma

Adenoid cystic carcinoma (ACC) is a relatively common salivary gland tumor, representing about 7.5% of carcinomas and 4% of all benign and malignant salivary gland neoplasms.[27] ACC is characterized by pronounced infiltration, often with perineural spread. Because of the slow growth of the tumor, about 70% of the patients are still alive after 5 years. However, frequent late recurrences and distant metastases to the lungs, bone, and liver decrease the 20-year survival rate to less than 20%.[53] ACC typically lacks the ability to metastasize to lymph nodes, preferring the hematogenous route.

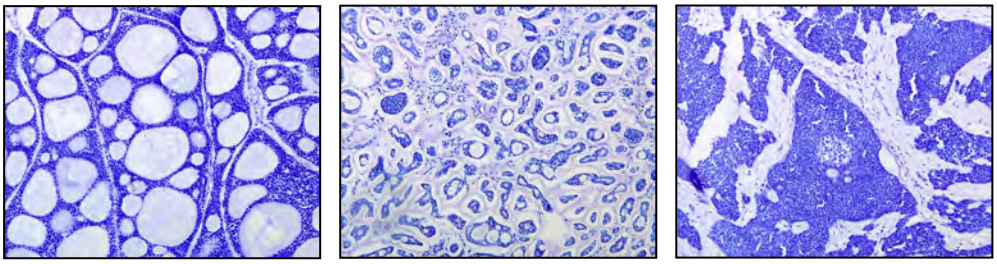

Figure 4 The three morphologic patterns in ACCs are depicted. On the left-hand side, the large pseudocystic structures of the cribriform subtype can be observed. In the middle figure, islands of tumor cells form the tubular pattern. On the right-hand side, the solid growth pattern can be seen (all 200x).

There are two types of neoplastic cells in this carcinoma. The majority are small basaloid myoepithelial-type cells and these are surrounded by scattered foci of intercalated-type cells.[54,55] Histologically, three different subtypes are recognized in ACC (Figure 4): (1) the classic **cribriform** type, exhibiting monomorphic cell islands with punched-out spaces containing glycosaminoglycans and basal lamina material produced by the tumor, giving rise to the 'Swiss cheese pattern'; (2) the **tubular** type, composed of ductal structures lined by two or more cell layers within a fibrous stroma, and (3) the **solid** type, which is composed of basaloid tumor cells and can contain foci of necrosis, cellular polymorphism, and mitoses. However, in most ACCs, two or more patterns are usually recognized and these tumors are classified according to the predominant pattern.[27,56]

Prognosis of ACC is mostly correlated with tumor stage, grade, anatomic site, status of surgical margins, and histologic pattern.[57,58] Regarding morphology, the solid subtype is considered as a high-grade lesion with high loss of heterozygosity (LOH) and reported recurrence rates of up to 100% compared to 50-80% for the tubular and cribriform variants.[59,60]

However, since the above-mentioned clinicopathological parameters are not unequivocal predictors of disease activity in ACC, investigations explored biomarkers on a molecular level. Using immunohistochemistry, expression levels of various markers have been evaluated, including proliferating cell nuclear antigen (PCNA),[61] epidermal growth factor receptor (EGFR),[44] HER-2/neu,[62] **Ki-67**,[63] **p53**, E-cadherin,[64] and **Cyclin D1**,[65] but results remain inconclusive. Only p53 proved to be a consistent marker of aggressiveness in ACC, as being highly expressed in the solid pattern,[66] correlating with unfavorable clinical outcome,[67,68] and having high LOH rates in the solid subtype.[69] High frequencies of promoter hypermethylation of E-cadherin and p16 have also been reported in ACC.[70,71]

Research on ACC tumorigenesis progressed further when chromosomal aberrations were examined by means of fluorescence in situ hybridization (FISH), LOH analysis, and microarray-based Comparative Genomic Hybridization (array CGH). These studies demonstrated frequent chromosome arm 6q, 12q, and 17p deletions,[72-74] a recurrent t(6;9)(q21-q25;p21-p22) translocation,[75] and LOH at

6q23-q25 in 76% of cases of ACC.[60] Generally, gains were found to be more common in ACC compared to losses, amongst which DNA copy number increases of 16p, 17q, 19 and 22q were the most prevalent.[76-78]

Regarding gene expression, recent oligonucleotide microarray analyses revealed a unique expression profile for ACC when compared to other common carcinomas.[79] Highly overexpressed genes encode for basement membrane and extracellular matrix proteins of myoepithelial differentiation, in addition to several transcription factors, like *SOX4* and *AP-2γ*, and members of the Wnt/β-catenin pathway.

Despite accumulating progress in understanding the molecular mechanisms underlying ACC tumorigenesis, there is still a paucity of data regarding the key genetic players.

3.2 Benign and Malignant Myoepithelioma

Tumor cells in benign and malignant myoepitheliomas (BMEs and MMEs, respectively) overwhelmingly demonstrate myoepithelial differentiation with only rare or absent ductal differentiation (Figure 5, left-hand picture).[1] Sheets and islands of myoepithelially differentiated cells are composed of various proportions of spindle, clear cell, plasmacytoid, and epithelioid cells. Most tumors are composed of a single cell type but combinations may occur. Because myoepitheliomas lack a luminal (ductal) component they may be mistaken for mesenchymal neoplasms.

The hypothesis that the constituent cell types in myoepitheliomas are representations of different stages in myoepithelial cell differentiation[80] is also reflected in their immunoprofiles. Epithelial differentiation is easily detectable, since all cell types are usually positive for cytokeratins 7 and 14.[81] Conversely, only the spindle cell type reacts strongly to smooth muscle actin (SMA) and muscle specific actin (MSA), demonstrating myogenous differentiation.[82] The absence of these myoid markers in the plasmacytoid cell type even encouraged Franquemont and Mills to reject this type as a true myoepithelioma.[82] Current theory comprises a spectrum of myoepithelial cell types, in which plasmacytoid cells have either lost or modified their ability to express myogenous markers.[83] Since extracellular matrix components are known to modulate morphogenetic changes and cytodifferentiation, they are suggested to be responsible for this process.[84]

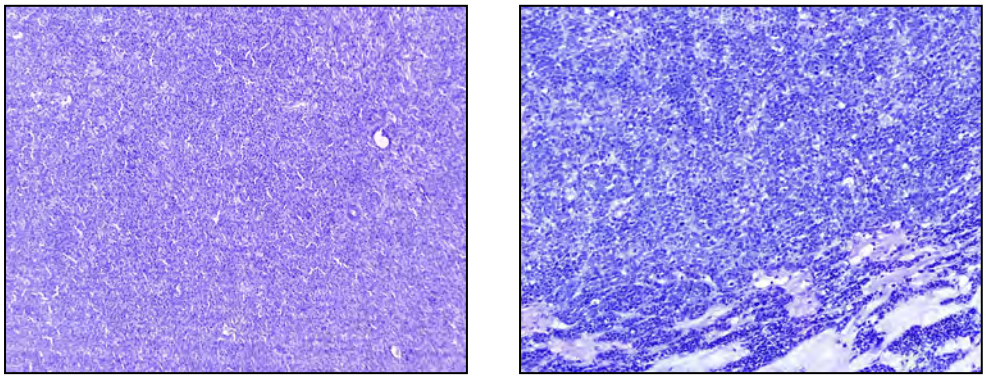

Figure 5 The left-hand haematoxylin & eosin staining depicts the histologic appearance of a benign myoepithelioma. Myoepithelial cells are uniformly distributed without a ductal component. On the right-hand side, polymorphous cells and diverse mitoses can be observed in a myoepithelial carcinoma (both 200x).

Although BMEs are biologically benign, they are reported to recur occasionally.[85] Malignant transformation, especially in long standing tumors or in tumors with multiple recurrences, has also been documented.[86] Myoepithelial carcinomas are more uncommon than their benign counterparts, representing less than 2% of all salivary gland tumors.[1] These carcinomas may arise *de novo*, but in most cases they develop in a pre-existing PA or from a BME, particularly in recurrences.[87-90] MMEs are locally aggressive salivary gland tumors, in which high **p53** and **Ki-67** expression is indicative of tumor recurrence and fatal patient outcome.[90,91] Carcinomas are differentiated from their benign counterparts by their characteristic multi-lobulated architecture, presence of infiltrating growth, necrotic areas, cellular polymorphism, and mitotic figures (Figure 5, right-side picture).[92,93]

Cytogenetic studies revealed a paucity of genomic aberrations, both in benign and the malignant myoepitheliomas.[94,95] The most frequent recurrent DNA copy number alterations in MMEs included gains of the chromosome arms 8q (26%) and 1q (21%), and of chromosome 5 (21%). BMEs display even less genomic events than MMEs, without recurrent aberrations.[96] Explanations for the lack of gross genomic alterations in these lesions might be that (1) alterations not detectable by array CGH, like balanced translocations or point mutations, are responsible for their tumorigenesis or that (2) myoepithelial cells have a greater ability to repair DNA.[10,94]

3.3 Carcinosarcoma

Carcinosarcomas (or **true malignant mixed tumors**) are rare neoplasms, representing 0.4% of all malignant salivary gland lesions.[97,98] By definition, they manifest both carcinomatous and sarcomatous elements (Figure 6).[1] The most frequent carcinomatous component is poorly differentiated adenocarcinoma or salivary duct carcinoma and the sarcomatous component is typically chondrosarcoma, however osteosarcoma and fibrosarcoma have also been identified.[27] Carcinosarcomas can develop within a PA[99,100] or appear *de novo*.[101] They are aggressive, high-grade neoplasms with a high mortality rate, due to local recurrences and distant metastases.[102,103]

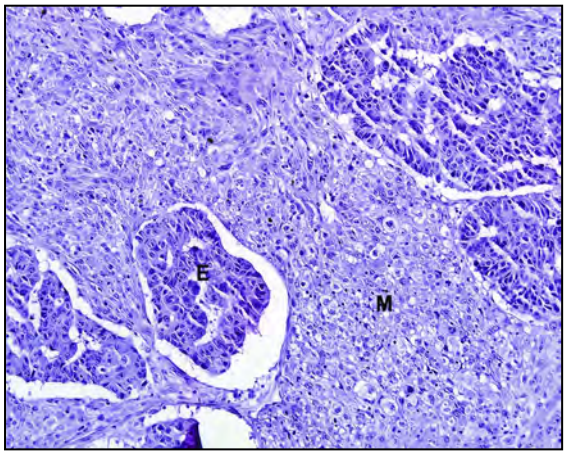

Figure 6 Histologic picture of a carcinosarcoma. Sheets of cells with epithelial differentiation **(E)** are surrounded by the sarcomatous tumor component **(M),** consisting of mesenchymally differentiated cells.

There is a long standing debate on the clonal composition and cellular origin of the two malignant components in carcinosarcomas.[104] Do the two morphologically different elements arise from the same precursor cell (i.e. **monoclonal origin**) or do they develop separately and represent a collision tumor (i.e. **polyclonal origin**)? Advocates of the latter hypothesis suggest two independent neoplastic processes in which the two histologically distinct malignant cell populations arise from separate cells (i.e. one epithelial, the other mesenchymal).[101] The former hypothesis defends the view that both components stem from the same transformed cell.[97,105] However, the type of cell designated as the common stem cell remains a matter of discussion.[99,101,106] Recent

research has shed some light on the different models of tumorigenesis. First, cytogenetic analyses revealed a high level of homology in chromosomal aberrations between the different components, suggesting a monoclonal origin.[107-110] Second, a combination of electron micrographic analysis and immunohistochemistry has indicated the most likely candidate for the common stem cell. Immunoreactivity for vimentin,[101,111] S-100 protein,[112] cytokeratin,[113] and calponin (Vékony *et al*. Oral Oncology 2008, *in press*) has been detected in both tumor elements, suggesting that carcinosarcomas are derived from the myoepithelium.[114,115] Electron microscopy observed organized and non-organized cytoplasmic filaments, indicative of at least rudimentary myoepithelial differentiation.[107] On the whole, accumulating data seem to support a monoclonal origin for the two components in carcinosarcoma with the myoepithelial cell as stem cell.

4 Cell cycle regulation and Tumorigenesis

Balanced regulation of cellular proliferation is critical for normal development and maintenance of tissues, and for allowing adaptation to changing circumstances. The pathogeneses of various human diseases are associated with the impaired function of components that control the capacity to maintain the numeric equilibrium. Disruption of the delicate homeostasis between cell growth and cell death can have disastrous consequences, as seen in human cancer.[116]

Mammalian cells have multiple safeguards to protect them against uncontrolled growth; however, sometimes this system fails. The process of tumorigenesis is initiated when a replication-competent cell acquires a mutation in a '**gatekeeping**' pathway that endows it with a selective growth advantage. From then on, a stepwise accumulation of genetic and epigenetic hits in three types of key regulatory genes are responsible for tumor formation: **oncogenes**, **tumor-suppressor genes**, and **stability genes**.[117] Oncogenes are mutated in a way that renders the gene constitutively active or active under conditions in which the wild-type gene is not. Oncogene activations can result from chromosomal translocations, gene amplifications or intragenic mutations. Tumor-suppressor genes are targeted in the opposite way: mutations reduce the activity of the gene product, mostly by mis-sense

mutations, deletions or epigenetic silencing. Stability genes or caretakers are responsible for repairing subtle mistakes made during normal DNA replication or induced by exposure to mutagens.

The phenotypic properties of cancer cells (i.e. self-sufficiency in growth signals, sustained angiogenesis, limitless replicative potential, evasion of apoptosis, and insensitivity to anti-growth signals) are the result of genetic and/or epigenetic alterations in a variety of molecules and growth regulatory pathways involved in cell cycle control. Most oncogenes and tumor-suppressor genes are directly or indirectly implicated in these critical cell cycle regulatory pathways (an integral part of the safeguard system); they are discussed in the next subchapters.

On the whole, gatekeeping mutations in particular cancers provide fundamental insights into their biology and pathogenesis and are of specific importance to future diagnostic and therapeutic strategies.

4.1 The Retinoblastoma Pathway

Following mitogen stimulation of quiescent cells, genes encoding D-type cyclins get activated at the beginning of the G1 phase.[118] **Cyclin D** then binds Cdk4 or Cdk6 to activate the kinase activity of these proteins. Among the cdk targets to become phosphorylated, the Retinoblastoma protein (pRb) is one of the best-studied examples. Rb is a central component of a transcriptional repression complex that inhibits the expression of multiple genes whose products are necessary for the G1/S transition and S phase (Figure 7, left-hand side). Active, hypophosphorylated Rb is able to bind proteins required for cell cycle progression, like the **E2F** transcription factors. Sequential phosphorylation of Rb by cdks produces a conformational change in the protein followed by the release of E2F, stimulating cellular proliferation. The E2F family of proteins comprises at least seven identified members with complex cell cycle and apoptotic activities.[119] Depending on the context, they regulate the expression of genes involved in cell cycle progression, DNA synthesis, checkpoint control, apoptosis, DNA repair, and development.

The gene locus *CDKN2a* encodes two intimately linked but distinct tumor-suppressor proteins, **p16^{INK4a}** and p14^{ARF} (also called p19^{ARF}

when referring specifically to the murine version), which act in two different pathways.[120] $P16^{INK4a}$ binds to Cdk4 and Cdk6, inhibiting their association with the D-type cyclins and subsequent phosphorylation of Rb. Thus, expression of $p16^{INK4a}$ maintains Rb in a hypophosphorylative state, promoting its binding to E2F and allowing G1 cell cycle arrest. On the other hand, $p14^{ARF}$ binds and inactivates the MDM2 protein (see section 4.2), preventing degradation of p53.

In vitro and *in vivo* induction of $P16^{INK4a}$ expression involves numerous DNA-damaging stimuli, including UV light, oxygen radicals, ionizing radiation, oncogenic stress, chemotherapeutic agents, and telomere dysfunction.[120] Activation of the locus is associated with the induction of senescence in most systems.

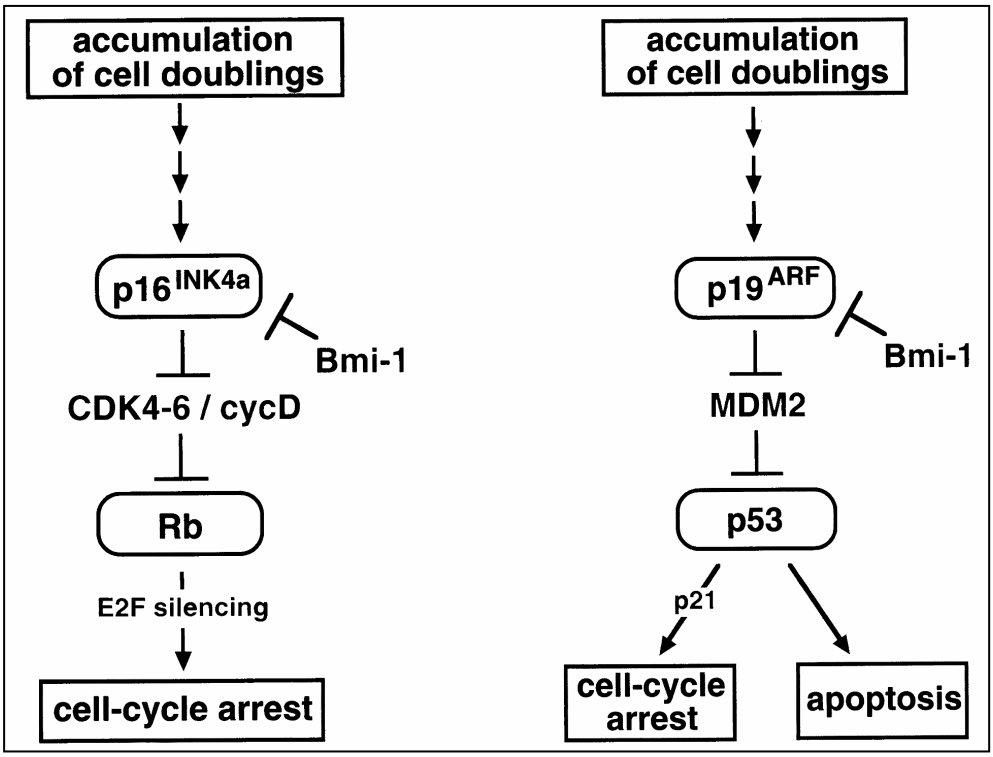

Figure 7 Schematic overview of the pRb and p53 pathways. $P19^{ARF}$ is the murine homologue of the human $p14^{ARF}$ gene. See text for details. (Source: Bringold F. *et al.* Exp Gerontology 2000)

4.2 The p53 Pathway

The nuclear phosphoprotein p53, or the '**guardian of the genome**', was the first tumor-suppressor gene to be identified.[121] In normal, healthy cells the p53 network is 'switched off'. Only when cells are stressed or damaged, p53 cellular level increases to initiate the transcription of genes involved in growth arrest, DNA repair, and/or apoptosis.[122] The amount of cellular p53 protein is determined by its degradation rather than by its synthesis. Responsible for the degradation is the MDM2 enzyme, which labels the p53 protein with ubiquitins and thus allows it to be detected by the proteosome (Figure 7, right-hand side).[123] Mutations of the *p53* gene, among the most frequent events in human cancer, frequently lead to the expression of an inactive protein with a prolonged half-life.[124] Because of this delayed degradation and consequent cellular accumulation, mutated p53 can be detected by immunohistochemistry.[125]

4.3 Altered Cell Cycle Control in Cancer

The key players of the pRb and p53 pathways have been found to be distorted in more than 80% of human neoplasms, either by mutations within the genes encoding the proteins or in their upstream regulators (reviewed in Michalides 1999 and Ortega *et al*. 2002).[126,127]

Overexpression of **Cyclin D1** prevents cells from entering quiescence and therefore accelerates cell proliferation. An important role for Cyclin D1 during malignant transformation has been shown in transgenic mice where Cyclin D1 cooperates with Myc in the induction of tumors and in parathyroid adenomas and in B-cell lymphomas, where its locus is rearranged. Amplification of *Cyclin D1* has been detected in various tumors and its overexpression has been shown to reduce mitogen requirement (reviewed in Coqueret 2002).[128]

Interestingly, deregulated **E2F1** expression can either promote or inhibit tumorigenesis depending on the cell type or the nature of other oncogenic mutations that are present.[129] Increased E2F1 levels have been found in several cancer cell lines and human tumors. In sharp contrast, reduced E2F1 expression in colon cancer, bladder cancer and

diffuse large B-cell lymphoma correlates with more aggressive disease.[130-132]

P16^{INK4a} is altered in a high percentage of human neoplasms.[133] This tumor-suppressor can be inactivated by a variety of mechanisms, including deletion, point mutation, and silencing by hypermethylation of genomic DNA. Interestingly, in tumors with p16 inactivated, pRb is always wild-type, whereas in tumors bearing pRb mutations, p16 is wild-type. This suggests that p16 and pRb act as a single functional unit in tumor suppression.[134]

As mentioned, **p53** alteration is a common event in human cancers. In addition to gene mutations, *p53* can also become inactivated by deletion, multiplication of the *MDM2* gene in the genome, deletion of p14^{ARF}, or mislocalization to the cytoplasm.[122]

4.4 Growth Factors

Normal cells require mitogenic growth signals before they can progress from a quiescent state into an active proliferative state. These signals are transmitted into the cell through an intracellular tyrosine kinase domain of growth factor receptors that bind distinctive classes of signaling molecules. Tumor cells generate many of their own growth signals, thereby reducing their dependence on stimulation from their normal tissue environment. One common strategy for a cancer cell is to synthesize a growth factor to which it is responsive, creating a positive feedback loop (i.e. autocrine stimulation). The production of platelet derived growth factor (**PDGF**) and tumor growth factor α (TGFα) by glioblastomas and sarcomas, respectively are two examples.[135] A different method employed by several tumors is to overexpress growth factor receptors and thus become hyper-responsive to levels of growth factors that normally would not trigger proliferation. For example, the epidermal growth factor receptor (**EGFR**) is upregulated in stomach, brain, and breast tumors.[136] Overexpression of another potent family of mitogens, the fibroblastic growth factors (**FGFs**), has also been linked to a variety of human malignancies, including salivary gland tumors.[137-139] A final way of acquiring self-sufficiency in growth signals consists of switching the type of growth factor receptor on the surface of a cancerous cell. An illustrative example of this is the progressive loss of

the keratinocyte growth factor receptor during malignant transformation of human salivary gland cells and the accompanying *de novo* expression of the fibroblastic growth factor receptor 1 and 4 genes (*FGFR1* and *FGFR4*, respectively).[140]

Developments during the last years have dramatically improved the interest for growth factors and their receptors as anti-cancer drug targets. Clinically successful approaches have been made to interfere with the signaling process by (1) specific monoclonal antibodies directed against the receptor or (2) via small molecules, preventing phosphorylation of the receptor upon ligand binding and thus blocking the function of the tyrosine kinase domain.[141] These therapeutic agents can be specifically directed against one receptor (e.g. Gefitinib) or have a broader range of receptor inhibition (Gleevec).[142-144]

5 Polycomb group proteins

The Polycomb group (PcG) genes have originally been identified in *Drosophila melanogaster* as epigenetic repressors of homeotic cluster genes based on mutant phenotypes.[145,146] During development, positions of the individual body parts of the fruit fly are defined by homeotic (so-called 'homeobox') gene expression. Mutations in PcG genes cause de-repression of specific homeobox members, leading to changes in the body plan. Thus, by regulating homeobox gene expression, PcG proteins ensure the maintenance of cell identity.

Extensive studies confirmed functional and evolutionary conservation of PcG genes among plants and mammals, underscoring their essential function for the development of multicellular organisms.[147,148]

At the molecular level, human PcG proteins are classified into two mutually exclusive groups on the basis of their association with distinct classes of multimeric complexes, termed **Polycomb repressive complexes** (PRCs).[149] In humans, the PRC1 (or 'maintenance complex') consists of the **BMI-1/MEL-18**, RING, HPC and HPH proteins. The PRC2 complex (or 'initiation complex') is composed of **EED**, the SET domain H3 lysine 27 methyltransferase **EZH2**, SUZ12, and YY1 (Figure 8).[146] These proteins represent the evolutionary conserved 'core', but the exact composition of PcG complexes is determined by cell and tissue

type and by proliferation status.[150] Furthermore, to exert their function several PcG proteins co-localize or interact with various non-PcG proteins, including CtBP, E2F6, and HDACs.[152,153]

Recent discovery of four different mammalian EED proteins interacting with EZH2 and SUZ12 revealed the existence of two other PcG complexes (PRC3 and PRC4) with differential histone substrate specificity.[151] EED1 is the largest protein and is primarily present in the PRC2 complex, whereas the two shortest forms (EED3 and EED4) are part of the PRC3 complex. The intermediate form EED2, is only present in undifferentiated pluripotent cells, as well as in cells that have lost their 'normal' regulation. Interestingly, overexpression of EZH2 has been recently shown to promote the formation of the PRC4 complex, containing EED2, in breast, colon and prostate cancers.[151]

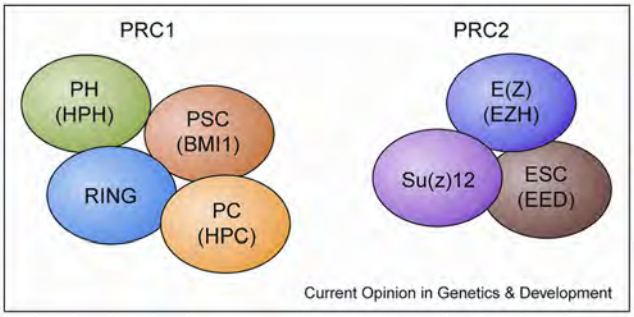

Figure 8 Overview of the core components in *Drosophila* and mammals (in brackets) of the two distinct PcG Repressive Complexes (PRCs). PRC1 include the PcG proteins PC (Polycomb), PH (Polyhomeotic), PSC (Posterior sex combs) and RING. The human homologues are HPC, HPH, BMI1 and RING, respectively. PRC2 comprises E(Z), Su(z)12 and Esc, with EZH, Su(z)12 and EED being their respective mammalian homologues. (Source: Otte, AP *et al.*, Curr Opinion Gen & Dev, 2003)

The mechanisms underlying the regulation of PcG target genes are not entirely understood, but extensive biochemical and genetic studies have provided a working model of how PcG proteins exert their effects (Figure 9). The PRCs are brought to the site of initial repression and act through epigenetic modification of chromatin structure to promote gene silencing.[154,155] More specifically, PRC2 is thought to initiate repression by removing the acetyl-mark on histone 3 lysine 27 (H3-K27) by HDAC proteins and replacing it by a triple methyl-group by EZH2 and SUZ12. In the presence of histone 1 (H1), PRC2 complex members methylate both H3K27 and H1K26.[151] Methylation of lysine residues is required to recruit the PRC1 complex, to allow its subsequent binding, which is

followed by BMI-1/RING-1A-mediated ubiquitylation of H2A-K119.[156,157,203] The interplay between these enzymatic activities facilitates a higher-order condensation of chromatin structure, and inhibition of chromatin remodeling activity in order to maintain silencing.

In addition to being essential in embryonic development, PcG proteins have also emerged as key players in stem cell renewal,[158,159] hematopoiesis,[160-162] and cell cycle progression.[163] Data obtained from murine lymphocytes revealed that the *p16*^{INK4a}/*p19*^{ARF} locus is a critical downstream target of Bmi-1. Recent analyses showed that several other PcG family members, such as CBX7, MEL-18 and RNF2, target the *CDKN2A* locus as well.[164,165] In humans however, the inverse correlation between BMI1 and p16^{INK4A} has not been consistently proven.[166,167] Furthermore, expression of the PRC2 genes EZH2 and EED has been shown to be suppressed by activated p53.[168,169] It was suggested that p53-mediated EZH2 suppression is one of the mechanisms by which p53 downregulates a large number of genes that encode proteins required for G2/M transition.[169]

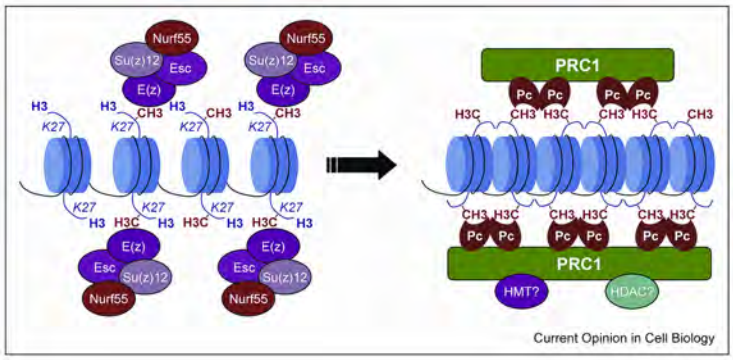

Figure 9 Suggested mechanism of action for PcG gene repression. In early stages of development and possibly also upon cell division, the PRC2 complex is recruited to PcG target genes to remove the acetylation mark on H3K27 and to trimethylate this lysine residue. Trimethylated K27 (or H1K26; not shown) then serves as an anchorage point for the Pc protein (human homologue HPC) and the PRC1 complex is recruited. HMT – histone methyltransferase; HDAC – histone deacetylase (Source: Lund A., *et al*. Curr Opinion Cell Biol 2004)

5.1 PcG proteins and Cancer

The role of PcG genes in the maintenance of cell identity is underscored by the fact that several PcG genes can be classified as oncogenes and

tumor-suppressor genes. In multiple malignancies, aberrant expression or recruitment of PcG proteins has been detected contributing to the reversion of normal cells to a more stem cell-like phenotype. Specific aspects of the mechanism underlying distorted PcG expression in malignancy remains to be elucidated, however it may partly be due to deregulation of the E2F/Rb pathway as E2F transcription factors bind and activate the promoter of many PRC component encoding genes.[153,168,170,171]

One of the most important discoveries is the induction of lymphomas in **Bmi-1** transgenic mice, where upregulation of *Bmi-1* results in downregulation of the cell cycle regulators $p16^{INK4a}$/$p19^{ARF}$.[172,173] Considering solid tumors, elevated *BMI-1* expression has been observed in breast, oral, and non small cell lung carcinomas.[174-176]

Not surprisingly, other PcG members have also been linked to tumorigenesis. **EZH2** is upregulated in several human tumors, such as multiple types of lymphoma, prostate and breast cancer.[177-180] Furthermore, *EZH2* expression is associated with increased tumor cell proliferation, poor prognosis, and aggressive tumor behavior.[181,182] However, conflicting results have also been reported in studies of prostate[183] and ovarian cancers[184], and it has been suggested that increased *EZH2* expression may be associated with a subset of tumors defined by high proliferation indices and not necessarily reflects a direct contribution of EZH2 to carcinogenesis and metastasis.[183,185]

Unlike Bmi-1, the closely related **Mel-18** is believed to have tumor-suppressive effects since Mel-18 inhibition enables immortal NIH 3T3 cells to form solid tumors in nude mice.[186] Conversely, overexpression of *Mel-18* in mice causes cell cycle arrest of B cells upon B cell receptor stimulation accompanied by the downregulation of c-myc expression.[187] Additionally, *MEL-18* was found to be downregulated in breast cancer cell lines.[188] To complicate the picture, the finding that MEL-18 and BMI-1 have overlapping functions in the proliferation of medulloblastoma cells suggests an oncogenic function for MEL-18.[189]

Finally, murine **Eed** has been shown to antagonize Bmi-1 and to have a repressive effect on hematopoietic cell proliferation.[190] Notably, human EED, together with EZH2 and SUZ12, is regulated by the E2F/Rb pathway and is required for cell proliferation.[168,191]

Taken together, the current findings provide evidence that PcG complexes are multifaceted, dynamic molecular modules exerting pleiotropic effects. Depending on context (i.e. cell type and developmental stage) the composition of PcG complexes and the role of their individual members can differ extensively.

6 Microarray-based Comparative Genomic Hybridization

Development of cancer is associated with the acquisition and progressive accumulation of genetic and epigenetic abnormalities, leading to aberrant expression of crucial proteins that ultimately lead to uncontrolled cell growth. As described in section 4, products of oncogenes and tumor-suppressors are among these crucial proteins and their respective amplification and deletion are common events in cancer initiation and progression.

Comparative genomic hybridization (CGH) is a molecular cytogenetic technique designed to comprehensively analyze the entire genome.[192] In a single experiment, it provides information on the relative DNA copy numbers of chromosome parts throughout the whole tumor genome. Microarray-based CGH (**array CGH**) is based on the principles of classical CGH, but the differentially labeled test and reference genomic DNA samples are hybridized to DNA targets arrayed on a glass slide rather than on metaphase chromosomes.[193] This results in a much higher sensitivity and spatial resolution, in which the resolution is defined by two factors, namely (1) the size of the nucleic acid targets and (2) the density of coverage over the genome. CGH arrays that use large-insert genomic clones (such as BAC and P1 artificial chromosomes) are able to detect single copy changes accurately and reliably.[194,195] The use of BACs with known map positions allows direct correlation of DNA copy number gains and losses with specific genomic sequence of known chromosomal locations.

Array CGH has rapidly become an important genome analysis tool in cancer research. Applications in cancer genetics include characterization of recurrent chromosomal gains and losses in a particular tumor type,[196] implication of specific genes in cancer development,[197] analysis of

progression,[198] the dissection of genetic changes in experimental models of carcinogenesis,[199] as well as subclassification and prognostic evaluation of cancer.[200,201] CGH also has potential clinical utility, for instance in the diagnosis of recurring chromosomal aberrations in various leukemias.[202]

Scope of the Thesis

In the present thesis, we attempted to get more insight in the disturbances underlying salivary gland tumors with myoepithelial differentiation. We have analyzed these tumors on two levels, (1) protein expression levels by immunohistochemistry and (2) genomic alterations by array CGH. We raised the following questions:

Chapter 2: What are the recurrent copy number changes in adenoid cystic carcinoma and do they correlate with patient and tumor characteristics?

We investigate the chromosomal copy numbers in a relatively large group of ACCs (*n*=18) by array CGH. Since loci of recurrent genomic aberrations are most likely to harbor genes contributing to ACC development, we examine the most frequent regions of chromosomal gains and losses for target genes. Additionally, we correlate our findings with tumor and patient characteristics to reveal genomic regions correlating with aggressive tumor behavior and survival.

Chapter 3: Are the two major 'safeguard' systems (i.e. pRb-p16 and p53 pathways) disturbed in ACC and can expression levels be used as prognostic indicators?

We explore the expression levels of the proteins $p16^{INK4a}$, E2F1, Cyclin D1, p53, and Ki-67 in 21 cases of paraffin-embedded ACC by immunohistochemical analysis. As a control group, we also determine the expression of the same proteins in normal salivary gland tissue (*n*=17). To investigate prognostic relevance of these gene products we correlate our results with patient and tumor characteristics.

Are PcG protein expression patterns altered in ACC compared with normal salivary gland tissue and do they have prognostic relevance?

By immunohistochemical analysis, we investigate the expression of the PcG proteins BMI-1, MEL-18, EZH2 and EED in normal salivary gland and tumor tissue. We perform statistical tests to evaluate the predictive value of PcG proteins in ACC.

Chapter 4: Which are the most frequent DNA copy number changes in benign and malignant myoepitheliomas?

In 15 paraffin-embedded BMEs and 12 paraffin-embedded MMEs, we determine chromosomal aberrations by array CGH and propose genes important for myoepithelial salivary gland tumor development. Also, we set out to identify patterns of DNA copy number alterations by unsupervised cluster analysis.

Chapter 5: Can we observe deregulation of the pRb-p16 and the p53 pathways and of the PcG proteins by detecting several of their members immunohistochemically?

We investigate the expression levels of the cell cycle regulators $p16^{INK4a}$, p53, Ki-67, E2F1, and Cyclin D1 in 49 paraffin-embedded BMEs, 30 paraffin-embedded MMEs and 5 paraffin-embedded histologically benign recurrences by immunohistochemistry, and compare our findings with protein levels in normal salivary gland tissue (n=17). In addition, we explore whether the mutually exclusive expression pattern of the two PcG complexes is still present in the benign and malignant tumors by detecting BMI-1, MEL-18 and EZH2 proteins immunohistochemically.

Chapter 6: Do the epithelial and mesenchymal components in a carcinosarcoma share a common precursor cell?

To uncover the chromosomal profiles of the two distinct histologic components, we extract genomic DNA from the paraffin-embedded tumor elements separately and hybridize them to a high-resolution oligonucleotide array. Next, we analyze the homology between the chromosomal alterations.

References

1. Barnes,L, Eveson,JW, Reichart,P et al. World Health Organization Classification of Tumours: Head and Neck Tumours. IARC*Press*, Lyon, 2005.
2. Martinez-Madrigal,F and Micheau,C. Histology of the major salivary glands. Am J Surg Pathol, 1989. 13(10):879-899.
3. Arey,LB. Developmental Anatomy. WB Saunders, Philadelphia, 1974.
4. Denny,PC, Ball,WD, and Redman,RS. Salivary glands: a paradigm for diversity of gland development. Crit Rev Oral Biol Med, 1997. 8(1):51-75.
5. Gasser,RF. The early development of the parotid gland around the facial nerve and its branches in man. Anat Rec, 1970. 167(1):63-77.
6. Bernier,JL and Bhaskar,SN. Lymphoepithelial lesions of salivary glands; histogenesis and classification based on 186 cases. Cancer, 1958. 11(6):1156-1179.
7. Jaskoll,T, Leo,T, Witcher,D et al. Sonic hedgehog signaling plays an essential role during embryonic salivary gland epithelial branching morphogenesis. Dev Dyn, 2004. 229(4):722-732.
8. Jaskoll,T, Witcher,D, Toreno,L et al. FGF8 dose-dependent regulation of embryonic submandibular salivary gland morphogenesis. Dev Biol, 2004. 268(2):457-469.
9. Hamperl,H. The myothelia (myoepithelial cells). Normal state; regressive changes; hyperplasia; tumors. Curr Top Pathol, 1970. 53161-220.
10. Lakhani,SR and O'Hare,MJ. The mammary myoepithelial cell--Cinderella or ugly sister? Breast Cancer Res, 2001. 3(1):1-4.
11. Archer,FL and Kao,VC. Immunohistochemical identification of actomyosin in myoepithelium of human tissues. Lab Invest, 1968. 18(6):669-674.
12. Tandler,B, Denning,CR, Mandel,ID et al. Ultrastructure of human labial salivary glands. 3. Myoepithelium and ducts. J Morphol, 1970. 130(2):227-245.
13. Caselitz,J, Loning,T, Staquet,MJ et al. Immunocytochemical demonstration of filamentous structures in the parotid gland. Occurrence of keratin and actin in normal and tumoral parotid gland with special respect to the myoepithelial cells. J Cancer Res Clin Oncol, 1981. 100(1):59-68.
14. Dardick,I, Parks,WR, Little,J et al. Characterization of cytoskeletal proteins in basal cells of human parotid salivary gland ducts. Virchows Arch A Pathol Anat Histopathol, 1988. 412(6):525-532.
15. Garrett,JR and Emmelin,N. Activities of salivary myoepithelial cells: a review. Med Biol, 1979. 57(1):1-28.
16. Guyton,AC. Anatomy and Physiology. Saunders College Publishing, New York, 1985.
17. Humphrey,SP and Williamson,RT. A review of saliva: normal composition, flow, and function. J Prosthet Dent, 2001. 85(2):162-169.
18. Eversole,LR. Histogenic classification of salivary tumors. Arch Pathol, 1971. 92(6):433-443.
19. Regezi,JA and Batsakis,JG. Histogenesis of salivary gland neoplasms. Otolaryngol Clin North Am, 1977. 10(2):297-307.
20. Batsakis,JG. Salivary gland neoplasia: an outcome of modified morphogenesis and cytodifferentiation. Oral Surg Oral Med Oral Pathol, 1980. 49(3):229-232.
21. Batsakis,JG, Regezi,JA, Luna,MA et al. Histogenesis of salivary gland neoplasms: a postulate with prognostic implications. J Laryngol Otol, 1989. 103(10):939-944.
22. Dardick,I and Burford-Mason,AP. Current status of histogenetic and morphogenetic concepts of salivary gland tumorigenesis. Crit Rev Oral Biol Med, 1993. 4(5):639-677.
23. Dardick,I, Byard,RW, and Carnegie,JA. A review of the proliferative capacity of major salivary glands and the relationship to current concepts of neoplasia in salivary glands. Oral Surg Oral Med Oral Pathol, 1990. 69(1):53-67.

24. Attie,JN and Sciubba,JJ. Tumors of Major and Minor Salivary Glands: Clinical and Pathologic Features. In: Current Problems in Surgery. Year Book Medical Publishers Inc.. Chicago. 1981. (Volume 18):80-81.
25. Dardick,I and Van Nostrand,AW. Morphogenesis of salivary gland tumors. A prerequisite to improving classification. Pathol Annu, 1987. 22 Pt 11-53.
26. Spiro,RH. Salivary neoplasms: overview of a 35-year experience with 2,807 patients. Head Neck Surg, 1986. 8(3):177-184.
27. Ellis,GL and Auclair,PL. Tumors of the Salivary Glands. Atlas of tumor pathology, 3rd series, fascicle 17. Washington D.C.: Armed Forces Institute of Pathology. 1996. Third fascicle
28. Rinaldo,A, Shaha,AR, Pellitteri,PK et al. Management of malignant sublingual salivary gland tumors. Oral Oncol, 2004. 40(1):2-5.
29. Lima,RA, Tavares,MR, Dias,FL et al. Clinical prognostic factors in malignant parotid gland tumors. Otolaryngol Head Neck Surg, 2005. 133(5):702-708.
30. Speight,PM and Barrett,AW. Salivary gland tumours. Oral Dis, 2002. 8(5):229-240.
31. Land,CE, Saku,T, Hayashi,Y et al. Incidence of salivary gland tumors among atomic bomb survivors, 1950-1987. Evaluation of radiation-related risk. Radiat Res, 1996. 146(1):28-36.
32. Sener,SF and Scanlon,EF. Irradiation induced salivary gland neoplasia. Ann Surg, 1980. 191(3):304-306.
33. Graham,S, Blanchet,M, and Rohrer,T. Cancer in asbestos-mining and other areas of Quebec. J Natl Cancer Inst, 1977. 59(4):1139-1145.
34. Horn-Ross,PL, Ljung,BM, and Morrow,M. Environmental factors and the risk of salivary gland cancer. Epidemiology, 1997. 8(4):414-419.
35. Swanson,GM and Belle,SH. Cancer morbidity among woodworkers in the U.S. automotive industry. J Occup Med, 1982. 24(4):315-319.
36. Swanson,GM and Burns,PB. Cancers of the salivary gland: workplace risks among women and men. Ann Epidemiol, 1997. 7(6):369-374.
37. Pinkston,JA and Cole,P. Cigarette smoking and Warthin's tumor. Am J Epidemiol, 1996. 144(2):183-187.
38. Terhaard,CH, Lubsen,H, Van der Tweel,I et al. Salivary gland carcinoma: independent prognostic factors for locoregional control, distant metastases, and overall survival: results of the Dutch head and neck oncology cooperative group. Head Neck, 2004. 26(8):681-692.
39. Therkildsen,MH, Christensen,M, Andersen,LJ et al. Salivary gland carcinomas--prognostic factors. Acta Oncol, 1998. 37(7-8):701-713.
40. Strick,MJ, Kelly,C, Soames,JV et al. Malignant tumours of the minor salivary glands--a 20 year review. Br J Plast Surg, 2004. 57(7):624-631.
41. Sung,MW, Kim,KH, Kim,JW et al. Clinicopathologic predictors and impact of distant metastasis from adenoid cystic carcinoma of the head and neck. Arch Otolaryngol Head Neck Surg, 2003. 129(11):1193-1197.
42. Pfeffer,MR, Talmi,Y, Catane,R et al. A phase II study of Imatinib for advanced adenoid cystic carcinoma of head and neck salivary glands. Oral Oncol, 2007. 43(1):33-36.
43. Lin,CH, Yen,RF, Jeng,YM et al. Unexpected rapid progression of metastatic adenoid cystic carcinoma during treatment with imatinib mesylate. Head Neck, 2005. 27(12):1022-1027.
44. Vered,M, Braunstein,E, and Buchner,A. Immunohistochemical study of epidermal growth factor receptor in adenoid cystic carcinoma of salivary gland origin. Head Neck, 2002. 24(7):632-636.
45. Younes,MN, Park,YW, Yazici,YD et al. Concomitant inhibition of epidermal growth factor and vascular endothelial growth factor receptor tyrosine kinases reduces growth and metastasis of human salivary adenoid cystic carcinoma in an orthotopic nude mouse model. Mol Cancer Ther, 2006. 5(11):2696-2705.

46. Agulnik,M, Cohen,EW, Cohen,RB et al. Phase II study of lapatinib in recurrent or metastatic epidermal growth factor receptor and/or erbB2 expressing adenoid cystic carcinoma and non adenoid cystic carcinoma malignant tumors of the salivary glands. J Clin Oncol, 2007. 25(25):3978-3984.
47. Sternlicht,MD and Barsky,SH. The myoepithelial defense: a host defense against cancer. Med Hypotheses, 1997. 48(1):37-46.
48. Barsky,SH. Myoepithelial mRNA expression profiling reveals a common tumor-suppressor phenotype. Exp Mol Pathol, 2003. 74(2):113-122.
49. Sternlicht,MD, Kedeshian,P, Shao,ZM et al. The human myoepithelial cell is a natural tumor suppressor. Clin Cancer Res, 1997. 3(11):1949-1958.
50. Sternlicht,MD, Safarians,S, Calcaterra,TC et al. Establishment and characterization of a novel human myoepithelial cell line and matrix-producing xenograft from a parotid basal cell adenocarcinoma. In Vitro Cell Dev Biol Anim, 1996. 32(9):550-563.
51. Nguyen,M, Lee,MC, Wang,JL et al. The human myoepithelial cell displays a multifaceted anti-angiogenic phenotype. Oncogene, 2000. 19(31):3449-3459.
52. Guelstein,VI, Tchypysheva,TA, Ermilova,VD et al. Myoepithelial and basement membrane antigens in benign and malignant human breast tumors. Int J Cancer, 1993. 53(2):269-277.
53. Spiro,RH, Huvos,AG, and Strong,EW. Adenoid cystic carcinoma of salivary origin. A clinicopathologic study of 242 cases. Am J Surg, 1974. 128(4):512-520.
54. Orenstein,JM, Dardick,I, and Van Nostrand,AW. Ultrastructural similarities of adenoid cystic carcinoma and pleomorphic adenoma. Histopathology, 1985. 9(6):623-638.
55. Chaudhry,AP, Leifer,C, Cutler,LS et al. Histogenesis of adenoid cystic carcinoma of the salivary glands. Light and electronmicroscopic study. Cancer, 1986. 58(1):72-82.
56. Batsakis,JG, Luna,MA, and El Naggar,A. Histopathologic grading of salivary gland neoplasms: III. Adenoid cystic carcinomas. Ann Otol Rhinol Laryngol, 1990. 99(12):1007-1009.
57. Szanto,PA, Luna,MA, Tortoledo,ME et al. Histologic grading of adenoid cystic carcinoma of the salivary glands. Cancer, 1984. 54(6):1062-1069.
58. Huang,M, Ma,D, Sun,K et al. Factors influencing survival rate in adenoid cystic carcinoma of the salivary glands. Int J Oral Maxillofac Surg, 1997. 26(6):435-439.
59. Tomich,CE. Surgical Pathology of the Salivary Glands. W.B. Saunders Philadelphia 1991. 333-349.
60. Stallmach,I, Zenklusen,P, Komminoth,P et al. Loss of heterozygosity at chromosome 6q23-25 correlates with clinical and histologic parameters in salivary gland adenoid cystic carcinoma. Virchows Arch, 2002. 440(1):77-84.
61. Cho,KJ, Lee,SS, and Lee,YS. Proliferating cell nuclear antigen and c-erbB-2 oncoprotein expression in adenoid cystic carcinomas of the salivary glands. Head Neck, 1999. 21(5):414-419.
62. Press,MF, Pike,MC, Hung,G et al. Amplification and overexpression of HER-2/neu in carcinomas of the salivary gland: correlation with poor prognosis. Cancer Res, 1994. 54(21):5675-5682.
63. Carlinfante,G, Lazzaretti,M, Ferrari,S et al. P53, bcl-2 and Ki-67 expression in adenoid cystic carcinoma of the palate. A clinico-pathologic study of 21 cases with long-term follow-up. Pathol Res Pract, 2005. 200(11-12):791-799.
64. Franchi,A, Gallo,O, Bocciolini,C et al. Reduced E-cadherin expression correlates with unfavorable prognosis in adenoid cystic carcinoma of salivary glands of the oral cavity. Am J Clin Pathol, 1999. 111(1):43-50.
65. Yasumatsu,R, Kuratomi,Y, Nakashima,T et al. Cyclin D1 expression does not effect cell proliferation in adenoid cystic carcinoma of the salivary gland. Eur Arch Otorhinolaryngol, 2004. 261(10):526-530.
66. Yamamoto,Y, Wistuba,II, Kishimoto,Y et al. DNA analysis at p53 locus in adenoid cystic carcinoma: comparison of molecular study and p53 immunostaining. Pathol Int, 1998. 48(4):273-280.

67. Cruz Perez,DE, de Abreu,AF, Nobuko,N, I et al. Prognostic factors in head and neck adenoid cystic carcinoma. Oral Oncol, 2006. 42(2):139-146.
68. Zhu,QR, White,FH, and Tipoe,GL. p53 oncoprotein accumulation in adenoid cystic carcinoma of parotid and palatine salivary glands. Pathology, 1997. 29(2):154-158.
69. Yamamoto,Y, Virmani,AK, Wistuba,II et al. Loss of heterozygosity and microsatellite alterations in p53 and RB genes in adenoid cystic carcinoma of the salivary glands. Hum Pathol, 1996. 27(11):1204-1210.
70. Zhang,CY, Mao,L, Li,L et al. Promoter methylation as a common mechanism for inactivating E-cadherin in human salivary gland adenoid cystic carcinoma. Cancer, 2007. 110(1):87-95.
71. Nishimine,M, Nakamura,M, Kishi,M et al. Alterations of p14ARF and p16INK4a genes in salivary gland carcinomas. Oncol Rep, 2003. 10(3):555-560.
72. Queimado,L, Reis,A, Fonseca,I et al. A refined localization of two deleted regions in chromosome 6q associated with salivary gland carcinomas. Oncogene, 1998. 16(1):83-88.
73. Roijer,E, Dahlenfors,R, Mark,J et al. Observations by chromosome banding, FISH and immunohistochemistry in an adenoid cystic carcinoma with del(17)(p13) as the sole deviation. Virchows Arch, 1997. 430(4):339-342.
74. El Rifai,W, Rutherford,S, Knuutila,S et al. Novel DNA copy number losses in chromosome 12q12--q13 in adenoid cystic carcinoma. Neoplasia, 2001. 3(3):173-178.
75. Nordkvist,A, Mark,J, Gustafsson,H et al. Non-random chromosome rearrangements in adenoid cystic carcinoma of the salivary glands. Genes Chromosomes Cancer, 1994. 10(2):115-121.
76. Vékony,H, Ylstra,B, Wilting,SM et al. DNA Copy Number Gains at Loci of Growth Factors and Their Receptors in Salivary Gland Adenoid Cystic Carcinoma. Clin Cancer Res, 2007. 13(11):3133-3139.
77. Freier,K, Flechtenmacher,C, Walch,A et al. Copy number gains on 22q13 in adenoid cystic carcinoma of the salivary gland revealed by comparative genomic hybridization and tissue microarray analysis. Cancer Genet Cytogenet, 2005. 159(1):89-95.
78. Kasamatsu,A, Endo,Y, Uzawa,K et al. Identification of candidate genes associated with salivary adenoid cystic carcinomas using combined comparative genomic hybridization and oligonucleotide microarray analyses. Int J Biochem Cell Biol, 2005. 37(9):1869-1880.
79. Frierson,HF, Jr., El Naggar,AK, Welsh,JB et al. Large scale molecular analysis identifies genes with altered expression in salivary adenoid cystic carcinoma. Am J Pathol, 2002. 161(4):1315-1323.
80. Da Silveira,EJ, Pereira,AL, Fontora,MC et al. Myoepithelioma of minor salivary gland--an immunohistochemical analysis of four cases. Rev Bras Otorrinolaringol (Engl Ed), 2006. 72(4):528-532.
81. Takai,Y, Dardick,I, Mackay,A et al. Diagnostic criteria for neoplastic myoepithelial cells in pleomorphic adenomas and myoepitheliomas. Immunocytochemical detection of muscle-specific actin, cytokeratin 14, vimentin, and glial fibrillary acidic protein. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1995. 79(3):330-341.
82. Franquemont,DW and Mills,SE. Plasmacytoid monomorphic adenoma of salivary glands. Absence of myogenous differentiation and comparison to spindle cell myoepithelioma. Am J Surg Pathol, 1993. 17(2):146-153.
83. Jaeger,RG, De Oliveira,PT, Jaeger,MM et al. Expression of smooth-muscle actin in cultured cells from human plasmacytoid myoepithelioma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1997. 84(6):663-667.
84. Jaeger,MM, Araujo,VC, Kachar,B et al. Effect of spatial arrangement of the basement membrane on cultured pleomorphic adenoma cells. Study by immunocytochemistry and electron and confocal microscopy. Virchows Arch, 1997. 430(6):467-477.

85. El Naggar,A, Batsakis,JG, Luna,MA et al. DNA content and proliferative activity of myoepitheliomas. J Laryngol Otol, 1989. 103(12):1192-1197.
86. Seifert,G. Histological Typing of Salivary Gland Tumors. World Health Organization, Springer-Verlag Berlin 1991.
87. Bombi,JA, Alos,L, Rey,MJ et al. Myoepithelial carcinoma arising in a benign myoepithelioma: immunohistochemical, ultrastructural, and flow-cytometrical study. Ultrastruct Pathol, 1996. 20(2):145-154.
88. Alos,L, Cardesa,A, Bombi,JA et al. Myoepithelial tumors of salivary glands: a clinicopathologic, immunohistochemical, ultrastructural, and flow-cytometric study. Semin Diagn Pathol, 1996. 13(2):138-147.
89. McCluggage,WG, Primrose,WJ, and Toner,PG. Myoepithelial carcinoma (malignant myoepithelioma) of the parotid gland arising in a pleomorphic adenoma. J Clin Pathol, 1998. 51(7):552-556.
90. Nagao,T, Sugano,I, Ishida,Y et al. Salivary gland malignant myoepithelioma: a clinicopathologic and immunohistochemical study of ten cases. Cancer, 1998. 83(7):1292-1299.
91. Rosa,JC, Felix,A, Fonseca,I et al. Immunoexpression of c-erbB-2 and p53 in benign and malignant salivary neoplasms with myoepithelial differentiation. J Clin Pathol, 1997. 50(8):661-663.
92. Dardick,I, Cavell,S, Boivin,M et al. Salivary gland myoepithelioma variants. Histological, ultrastructural, and immunocytological features. Virchows Arch A Pathol Anat Histopathol, 1989. 416(1):25-42.
93. Darvishian,F and Lin,O. Myoepithelial cell-rich neoplasms: cytologic features of benign and malignant lesions. Cancer, 2004. 102(6):355-361.
94. Hungermann,D, Roeser,K, Buerger,H et al. Relative paucity of gross genetic alterations in myoepitheliomas and myoepithelial carcinomas of salivary glands. J Pathol, 2002. 198(4):487-494.
95. Martins,C, Fonseca,I, Felix,A et al. Benign salivary gland tumors: a cytogenetic study of 21 cases. J Surg Oncol, 1995. 60(4):232-237.
96. El Naggar,AK, Lovell,M, Callender,DL et al. Cytogenetic analysis of a primary salivary gland myoepithelioma. Cancer Genet Cytogenet, 1999. 113(1):49-53.
97. Stephen,J, Batsakis,JG, Luna,MA et al. True malignant mixed tumors (carcinosarcoma) of salivary glands. Oral Surg Oral Med Oral Pathol, 1986. 61(6):597-602.
98. Gnepp,DR. Malignant mixed tumors of the salivary glands: a review. Pathol Annu, 1993. 28 Pt 1279-328.
99. Gandour-Edwards,RF, Donald,PJ, Vogt,PJ et al. Carcinosarcoma (malignant mixed tumor) of the parotid: report of a case with a pure rhabdomyosarcoma component. Head Neck, 1994. 16(4):379-382.
100. Kwon,MY and Gu,M. True malignant mixed tumor (carcinosarcoma) of parotid gland with unusual mesenchymal component: a case report and review of the literature. Arch Pathol Lab Med, 2001. 125(6):812-815.
101. Bleiweiss,IJ, Huvos,AG, Lara,J et al. Carcinosarcoma of the submandibular salivary gland. Immunohistochemical findings. Cancer, 1992. 69(8):2031-2035.
102. Tortoledo,ME, Luna,MA, and Batsakis,JG. Carcinomas ex pleomorphic adenoma and malignant mixed tumors. Histomorphologic indexes. Arch Otolaryngol, 1984. 110(3):172-176.
103. Wahlberg,P, Anderson,H, Biorklund,A et al. Carcinoma of the parotid and submandibular glands--a study of survival in 2465 patients. Oral Oncol, 2002. 38(7):706-713.
104. Wick,MR and Swanson,PE. Carcinosarcomas: current perspectives and an historical review of nosological concepts. Semin Diagn Pathol, 1993. 10(2):118-127.
105. Carson,HJ, Tojo,DP, Chow,JM et al. Carcinosarcoma of salivary glands with unusual stromal components. Report of two cases and review of the literature. Oral Surg Oral Med Oral Pathol Oral Radiol Endod, 1995. 79(6):738-746.

106. Latkovich,P and Johnson,RL. Carcinosarcoma of the parotid gland: report of a case with cytohistologic and immunohistochemical findings. Arch Pathol Lab Med, 1998. 122(8):743-746.
107. Gotte,K, Riedel,F, Coy,JF et al. Salivary gland carcinosarcoma: immunohistochemical, molecular genetic and electron microscopic findings. Oral Oncol, 2000. 36(4):360-364.
108. Torenbeek,R, Hermsen,MA, Meijer,GA et al. Analysis by comparative genomic hybridization of epithelial and spindle cell components in sarcomatoid carcinoma and carcinosarcoma: histogenetic aspects. J Pathol, 1999. 189(3):338-343.
109. Fowler,MH, Fowler,J, Ducatman,B et al. Malignant mixed tumors of the salivary gland: a study of loss of heterozygosity in tumor suppressor genes. Mod Pathol, 2006. 19(3):350-355.
110. Adam,P, Zettl,A, Zollner,U et al. Metastasizing vulvar carcinosarcoma with squamous carcinomatous and leiomyosarcomatous differentiation: genetic evidence of clonal origin. Hum Pathol, 2005. 36(10):1143-1147.
111. Takata,T, Nikai,H, Ogawa,I et al. Ultrastructural and immunohistochemical observations of a true malignant mixed tumor (carcinosarcoma) of the tongue. J Oral Pathol Med, 1990. 19(6):261-265.
112. Takata,T, Nikai,H, Ogawa,I et al. Ultrastructural and immunohistochemical observations of a true malignant mixed tumor (carcinosarcoma) of the tongue. J Oral Pathol Med, 1990. 19(6):261-265.
113. Hellquist,H and Michaels,L. Malignant mixed tumour. A salivary gland tumour showing both carcinomatous and sarcomatous features. Virchows Arch A Pathol Anat Histopathol, 1986. 409(1):93-103.
114. Takata,T, Nikai,H, Ogawa,I et al. Ultrastructural and immunohistochemical observations of a true malignant mixed tumor (carcinosarcoma) of the tongue. J Oral Pathol Med, 1990. 19(6):261-265.
115. Dardick,I, Hardie,J, Thomas,MJ et al. Ultrastructural contributions to the study of morphological differentiation in malignant mixed (pleomorphic) tumors of salivary gland. Head Neck, 1989. 11(1):5-21.
116. Hanahan,D and Weinberg,RA. The hallmarks of cancer. Cell, 2000. 100(1):57-70.
117. Renan,MJ. How many mutations are required for tumorigenesis? Implications from human cancer data. Mol Carcinog, 1993. 7(3):139-146.
118. Johnson,DG and Walker,CL. Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol, 1999. 39295-312.
119. Bracken,AP, Ciro,M, Cocito,A et al. E2F target genes: unraveling the biology. Trends Biochem Sci, 2004. 29(8):409-417.
120. Kim,WY and Sharpless,NE. The regulation of INK4/ARF in cancer and aging. Cell, 2006. 127(2):265-275.
121. DeLeo,AB, Jay,G, Appella,E et al. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. Proc Natl Acad Sci U S A, 1979. 76(5):2420-2424.
122. Vogelstein,B, Lane,D, and Levine,AJ. Surfing the p53 network. Nature, 2000. 408(6810):307-310.
123. Momand,J, Wu,HH, and Dasgupta,G. MDM2--master regulator of the p53 tumor suppressor protein. Gene, 2000. 242(1-2):15-29.
124. Hollstein,M, Sidransky,D, Vogelstein,B et al. p53 mutations in human cancers. Science, 1991. 253(5015):49-53.
125. Oren,M. The p53 cellular tumor antigen: gene structure, expression and protein properties. Biochim Biophys Acta, 1985. 823(1):67-78.
126. Michalides,RJ. Cell cycle regulators: mechanisms and their role in aetiology, prognosis, and treatment of cancer. J Clin Pathol, 1999. 52(8):555-568.
127. Ortega,S, Malumbres,M, and Barbacid,M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim Biophys Acta, 2002. 1602(1):73-87.

128. Coqueret,O. Linking cyclins to transcriptional control. Gene, 2002. 299(1-2):35-55.
129. Johnson,DG and Degregori,J. Putting the Oncogenic and Tumor Suppressive Activities of E2F into Context. Curr Mol Med, 2006. 6(7):731-738.
130. Rabbani,F, Richon,VM, Orlow,I et al. Prognostic significance of transcription factor E2F-1 in bladder cancer: genotypic and phenotypic characterization. J Natl Cancer Inst, 1999. 91(10):874-881.
131. Moller,MB, Kania,PW, Ino,Y et al. Frequent disruption of the RB1 pathway in diffuse large B cell lymphoma: prognostic significance of E2F-1 and p16INK4A. Leukemia, 2000. 14(5):898-904.
132. Bramis,J, Zacharatos,P, Papaconstantinou,I et al. E2F-1 transcription factor immunoexpression is inversely associated with tumor growth in colon adenocarcinomas. Anticancer Res, 2004. 24(5A):3041-3047.
133. Kamb,A, Gruis,NA, Weaver-Feldhaus,J et al. A cell cycle regulator potentially involved in genesis of many tumor types. Science, 1994. 264(5157):436-440.
134. Parry,D, Bates,S, Mann,DJ et al. Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4/MTS1 tumour suppressor gene product. EMBO J, 1995. 14(3):503-511.
135. Fedi,P, Tronick,SR, and Aaronson,SA. Growth factors. Cancer medicine. Williams and Wilkins Baltimore, MD. 1997. 41-64.
136. Yarden,Y and Ullrich,A. Growth factor receptor tyrosine kinases. Annu Rev Biochem, 1988. 57443-478.
137. Myoken,Y, Myoken,Y, Okamoto,T et al. Immunohistochemical study of overexpression of fibroblast growth factor-1 (FGF-1), FGF-2, and FGF receptor-1 in human malignant salivary gland tumours. J Pathol, 1996. 178(4):429-436.
138. Myoken,Y, Myoken,Y, Okamoto,T et al. Immunohistochemical localization of fibroblast growth factor-1 (FGF-1), FGF-2 and fibroblast growth factor receptor-1 (FGFR-1) in pleomorphic adenoma of the salivary glands. J Oral Pathol Med, 1997. 26(1):17-22.
139. Yura,Y, Yoshioka,Y, Yamamoto,S et al. Enhancing effects of fibroblast growth factor on the proliferation of salivary gland carcinoma cells and salivary gland carcinogenesis. J Oral Pathol Med, 2001. 30(3):159-167.
140. Zhang,Y, Wang,H, Toratani,S et al. Growth inhibition by keratinocyte growth factor receptor of human salivary adenocarcinoma cells through induction of differentiation and apoptosis. Proc Natl Acad Sci U S A, 2001. 98(20):11336-11340.
141. Bianco,R, Melisi,D, Ciardiello,F et al. Key cancer cell signal transduction pathways as therapeutic targets. Eur J Cancer, 2006. 42(3):290-294.
142. Harari,PM. Epidermal growth factor receptor inhibition strategies in oncology. Endocr Relat Cancer, 2004. 11(4):689-708.
143. Manetti,F and Botta,M. Small-molecule inhibitors of fibroblast growth factor receptor (FGFR) tyrosine kinases (TK). Curr Pharm Des, 2003. 9(7):567-581.
144. Baselga,J. Targeting tyrosine kinases in cancer: the second wave. Science, 2006. 312(5777):1175-1178.
145. Simon,J. Locking in stable states of gene expression: transcriptional control during Drosophila development. Curr Opin Cell Biol, 1995. 7(3):376-385.
146. Ringrose,L and Paro,R. Epigenetic Regulation of Cellular Memory by the Polycomb and Trithorax Group Proteins. Annu Rev Genet, 2004.
147. Muller,J, Gaunt,S, and Lawrence,PA. Function of the Polycomb protein is conserved in mice and flies. Development, 1995. 121(9):2847-2852.
148. Sorensen,MB, Chaudhury,AM, Robert,H et al. Polycomb group genes control pattern formation in plant seed. Curr Biol, 2001. 11(4):277-281.
149. Satijn,DP and Otte,AP. Polycomb group protein complexes: do different complexes regulate distinct target genes? Biochim Biophys Acta, 1999. 1447(1):1-16.
150. Gunster,MJ, Raaphorst,FM, Hamer,KM et al. Differential expression of human Polycomb group proteins in various tissues and cell types. J Cell Biochem Suppl, 2001. Suppl 36129-143.

151. Kuzmichev,A, Margueron,R, Vaquero,A et al. Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation. Proc Natl Acad Sci U S A, 2005. 102(6):1859-1864.
152. Van der Vlag,J and Otte,AP. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. Nat Genet, 1999. 23(4):474-478.
153. Trimarchi,JM, Fairchild,B, Wen,J et al. The E2F6 transcription factor is a component of the mammalian Bmi1-containing polycomb complex. Proc Natl Acad Sci U S A, 2001. 98(4):1519-1524.
154. Pirrotta,V. Polycombing the genome: PcG, trxG, and chromatin silencing. Cell, 1998. 93(3):333-336.
155. Levine,SS, King,IF, and Kingston,RE. Division of labor in polycomb group repression. Trends Biochem Sci, 2004. 29(9):478-485.
156. Czermin,B, Melfi,R, McCabe,D et al. Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell, 2002. 111(2):185-196.
157. Muller,J, Hart,CM, Francis,NJ et al. Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell, 2002. 111(2):197-208.
158. Raaphorst,FM. Self-renewal of hematopoietic and leukemic stem cells: a central role for the Polycomb-group gene Bmi-1. Trends Immunol, 2003. 24(10):522-524.
159. Valk-Lingbeek,ME, Bruggeman,SW, and Van Lohuizen,M. Stem cells and cancer; the polycomb connection. Cell, 2004. 118(4):409-418.
160. Raaphorst,FM, Otte,AP, Van Kemenade,FJ et al. Distinct BMI-1 and EZH2 expression patterns in thymocytes and mature T cells suggest a role for Polycomb genes in human T cell differentiation. J Immunol, 2001. 166(10):5925-5934.
161. Lessard,J, Baban,S, and Sauvageau,G. Stage-specific expression of polycomb group genes in human bone marrow cells. Blood, 1998. 91(4):1216-1224.
162. Raaphorst,FM, Otte,AP, and Meijer,CJ. Polycomb-group genes as regulators of mammalian lymphopoiesis. Trends Immunol, 2001. 22(12):682-690.
163. Dahiya,A, Wong,S, Gonzalo,S et al. Linking the Rb and polycomb pathways. Mol Cell, 2001. 8(3):557-569.
164. Voncken,JW, Roelen,BA, Roefs,M et al. Rnf2 (Ring1b) deficiency causes gastrulation arrest and cell cycle inhibition. Proc Natl Acad Sci U S A, 2003. 100(5):2468-2473.
165. Gil,J, Bernard,D, Martinez,D et al. Polycomb CBX7 has a unifying role in cellular lifespan. Nat Cell Biol, 2004. 6(1):67-72.
166. Silva,J, García,JM, Peña,C el al. Implication of polycomb members Bmi-1, Mel-18, and Hpc-2 in the regulation of p16INK4a, p14ARF, h-TERT, and c-Myc expression in primary breast carcinomas. Clin Cancer Res, 2006. 12(23):6929-6936.
167. Douglas,D, Hsu,JH, Hung,L et al. BMI-1 promotes ewing sarcoma tumorigenicity independent of CDKN2A repression. Cancer Res, 2008. 68(16):6507-6515.
168. Bracken,AP, Pasini,D, Capra,M et al. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J, 2003. 22(20):5323-5335.
169. Tang,X, Milyavsky,M, Shats,I et al. Activated p53 suppresses the histone methyltransferase EZH2 gene. Oncogene, 2004. 23(34):5759-5769.
170. Attwooll,C, Oddi,S, Cartwright,P et al. A novel repressive E2F6 complex containing the polycomb group protein, EPC1, that interacts with EZH2 in a proliferation-specific manner. J Biol Chem, 2005. 280(2):1199-1208.
171. Nowak,K, Kerl,K, Fehr,D et al. BMI1 is a target gene of E2F-1 and is strongly expressed in primary neuroblastomas. Nucleic Acids Res, 2006. 34(6):1745-1754.
172. Jacobs,JJ, Kieboom,K, Marino,S et al. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature, 1999. 397(6715):164-168.

173. Jacobs,JJ, Scheijen,B, Voncken,JW et al. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev, 1999. 13(20):2678-2690.
174. Silva,J, Garcia,JM, Pena,C et al. Implication of polycomb members Bmi-1, Mel-18, and Hpc-2 in the regulation of p16INK4a, p14ARF, h-TERT, and c-Myc expression in primary breast carcinomas. Clin Cancer Res, 2006. 12(23):6929-6936.
175. Breuer,RH, Snijders,PJ, Sutedja,GT et al. Expression of the p16(INK4a) gene product, methylation of the p16(INK4a) promoter region and expression of the polycomb-group gene BMI-1 in squamous cell lung carcinoma and premalignant endobronchial lesions. Lung Cancer, 2005. 48(3):299-306.
176. Kang,MK, Kim,RH, Kim,SJ et al. Elevated Bmi-1 expression is associated with dysplastic cell transformation during oral carcinogenesis and is required for cancer cell replication and survival. Br J Cancer, 2007. 96(1):126-133.
177. Van Leenders,GJ, Dukers,D, Hessels,D et al. Polycomb-Group Oncogenes EZH2, BMI1, and RING1 Are Overexpressed in Prostate Cancer With Adverse Pathologic and Clinical Features. Eur Urol, 2006.
178. Raaphorst,FM, Meijer,CJ, Fieret,E et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia, 2003. 5(6):481-488.
179. Dukers,DF, Van Galen,JC, Giroth,C et al. Unique polycomb gene expression pattern in Hodgkin's lymphoma and Hodgkin's lymphoma-derived cell lines. Am J Pathol, 2004. 164(3):873-881.
180. Varambally,S, Dhanasekaran,SM, Zhou,M et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature, 2002. 419(6907):624-629.
181. Collett,K, Eide,GE, Arnes,J et al. Expression of enhancer of zeste homologue 2 is significantly associated with increased tumor cell proliferation and is a marker of aggressive breast cancer. Clin Cancer Res, 2006. 12(4):1168-1174.
182. Bachmann,IM, Halvorsen,OJ, Collett,K et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol, 2006. 24(2):268-273.
183. Lapointe,J, Li,C, Higgins,JP et al. Gene expression profiling identifies clinically relevant subtypes of prostate cancer. Proc Natl Acad Sci U S A, 2004. 101(3):811-816.
184. Adib,TR, Henderson,S, Perrett,C et al. Predicting biomarkers for ovarian cancer using gene-expression microarrays. Br J Cancer, 2004. 90(3):686-692.
185. Sellers,WR and Loda,M. The EZH2 polycomb transcriptional repressor--a marker or mover of metastatic prostate cancer? Cancer Cell, 2002. 2(5):349-350.
186. Kanno,M, Hasegawa,M, Ishida,A et al. mel-18, a Polycomb group-related mammalian gene, encodes a transcriptional negative regulator with tumor suppressive activity. EMBO J, 1995. 14(22):5672-5678.
187. Tetsu,O, Ishihara,H, Kanno,R et al. mel-18 negatively regulates cell cycle progression upon B cell antigen receptor stimulation through a cascade leading to c-myc/cdc25. Immunity, 1998. 9(4):439-448.
188. Matsuo,F, Yano,K, Saito,H et al. Mutation analysis of the mel-18 gene that shows decreased expression in human breast cancer cell lines. Breast Cancer, 2002. 9(1):33-38.
189. Wiederschain,D, Chen,L, Johnson,B et al. Contribution of polycomb homologues Bmi-1 and Mel-18 to medulloblastoma pathogenesis. Mol Cell Biol, 2007. 27(13):4968-4979.
190. Lessard,J, Schumacher,A, Thorsteinsdottir,U et al. Functional antagonism of the Polycomb-Group genes eed and Bmi1 in hemopoietic cell proliferation. Genes Dev, 1999. 13(20):2691-2703.
191. Pasini,D, Bracken,AP, Jensen,MR et al. Suz12 is essential for mouse development and for EZH2 histone methyltransferase activity. EMBO J, 2004. 23(20):4061-4071.
192. Kallioniemi,A, Kallioniemi,OP, Sudar,D et al. Comparative genomic hybridization for molecular cytogenetic analysis of solid tumors. Science, 1992. 258(5083):818-821.

193. Pinkel,D, Segraves,R, Sudar,D et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet, 1998. 20(2):207-211.
194. Pinkel,D and Albertson,DG. Array comparative genomic hybridization and its applications in cancer. Nat Genet, 2005. 37 SupplS11-S17.
195. Coe,BP, Ylstra,B, Carvalho,B et al. Resolving the resolution of array CGH. Genomics, 2007. 89(5):647-653.
196. Larramendy,ML, Kaur,S, Svarvar,C et al. Gene copy number profiling of soft-tissue leiomyosarcomas by array-comparative genomic hybridization. Cancer Genet Cytogenet, 2006. 169(2):94-101.
197. Okamoto,H, Yasui,K, Zhao,C et al. PTK2 and EIF3S3 genes may be amplification targets at 8q23-q24 and are associated with large hepatocellular carcinomas. Hepatology, 2003. 38(5):1242-1249.
198. Paris,PL, Andaya,A, Fridlyand,J et al. Whole genome scanning identifies genotypes associated with recurrence and metastasis in prostate tumors. Hum Mol Genet, 2004. 13(13):1303-1313.
199. Van de Wiel,MA, Costa,JL, Smid,K et al. Expression microarray analysis and oligo array comparative genomic hybridization of acquired gemcitabine resistance in mouse colon reveals selection for chromosomal aberrations. Cancer Res, 2005. 65(22):10208-10213.
200. Blaveri,E, Brewer,JL, Roydasgupta,R et al. Bladder cancer stage and outcome by array-based comparative genomic hybridization. Clin Cancer Res, 2005. 11(19 Pt 1):7012-7022.
201. Weiss,MM, Kuipers,EJ, Postma,C et al. Genomic profiling of gastric cancer predicts lymph node status and survival. Oncogene, 2003. 22(12):1872-1879.
202. Kristensen,TD, Wesenberg,F, Jonsson,OG et al. High-resolution comparative genomic hybridisation yields a high detection rate of chromosomal aberrations in childhood acute lymphoblastic leukaemia. Eur J Haematol, 2003. 70(6):363-372.
203. Cao,R, Tsukada,Y, Zhang,Y. Role of Bmi-1 and RING1A in H2A ubiquitylation and Hox gene silencing. Molecular Cell, 2005. 20(6): 845-854.

CHAPTER 2

DNA Copy Number Gains at Loci of Growth Factors and Their Receptors in Salivary Gland Adenoid Cystic Carcinoma

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Clinical Cancer Research 2007; 13(11): 3133-3139.

Chapter 2

ABSTRACT

Adenoid cystic carcinoma (ACC) is a malignant salivary gland tumor with a high mortality rate due to late, distant metastases. This study aimed at unraveling common genetic abnormalities associated with ACC. Additionally, chromosomal changes were correlated with patient characteristics and survival. Microarray-based comparative genomic hybridization was done to a series of 18 paraffin-embedded primary ACCs using a genome-wide scanning BAC array. A total of 238 aberrations were detected, representing more gains than losses (205 vs. 33, respectively). Most frequent gains (>60%) were observed at 9q33.3-q34.3, 11q13.3, 11q23.3, 19p13.3-p13.11, 19q12-q13.43, 21q22.3, and 22q13.33. These loci harbor numerous growth factor [fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF)] and growth factor receptor (FGFR3 and PDGFRβ) genes. Gains at the FGF(R) regions occurred significantly more frequently in the recurred/metastasized ACCs compared with indolent ACCs. Furthermore, patients with 17 or more chromosomal aberrations had a significantly less favorable outcome than patients with fewer chromosomal aberrations (log rank=5.2; P=0.02). In conclusion, frequent DNA copy number gains at loci of growth factors and their receptors suggest their involvement in ACC initiation and progression. Additionally, the presence of FGFR3 and PDGFRβ in increased chromosomal regions suggests a possible role for autocrine stimulation in ACC tumorigenesis.

INTRODUCTION

Adenoid cystic carcinoma (ACC) is one of the most common malignant tumors of the salivary glands. The tumor consists of epithelial and myoepithelial cells and is characterized by slow growth, multiple late recurrences, and distant metastases, mostly to the lungs, bone, and liver.[1] The neoplasm rarely metastasizes to lymph nodes, preferring the hematogenous route. Furthermore, ACC shows pronounced infiltration, often with perineural spread.

Three different histologic subtypes have been described: the cribriform type (1), exhibiting monomorphic cell islands with punched-out spaces, causing the “Swiss cheese pattern”; the tubular type (2), composed of ductal structures lined by two or more cell layers within a fibrous stroma; and the solid type (3), which is composed of basaloid tumor cells and can contain foci of necrosis, cellular polymorphism, and mitoses. However, in most ACCs, two or more patterns are recognized.

With respect to chromosomal alterations in ACC, fluorescence *in situ* hybridization and loss of heterozygosity (LOH) analysis studies[2-7] have shown frequent 6q and 17p deletions and a recurrent t(6;9)(q21-q25;p21-p22) translocation. Immunohistochemistry has implied various proteins as molecular markers of progression in ACC, but results were inconclusive.[8-12] Only p53 proved to be a consistent marker of aggressiveness in ACC, being highly expressed in the solid pattern,[13] correlating with unfavorable clinical outcome[14] and having high LOH rates.[15] Recent oligonucleotide microarray analyses revealed a unique expression profile for ACC when compared with other common carcinomas. Genes that are highly overexpressed in this tumor include several transcription factors, such as SOX4 and AP-2γ, and members of the Wnt/β-catenin pathway.[16,17] Until now, copy number alterations in ACC have only been investigated by classic comparative genomic hybridization (CGH) with a resolution of 5 Mb or more[18] and results have never been correlated to patient data. Although yielding valuable information, further drawbacks of these studies are a small sample size and the limited resolution of the technique. Gains were detected far more frequently in ACC (e.g., on 22q13) than losses, but a recurrent loss in chromosome 12q12-q13 has also been observed.[19-22]

The present study combines the use of microarray-based CGH (array CGH) with a relatively large number of tumor specimens. Array CGH allows the use of archival material[23] and provides a high resolution and sensitivity, which allows for the detection of small chromosomal copy number changes.[24]

To detect genetic abnormalities that may identify loci of genes involved in the development of ACC, we investigated the chromosomal aberrations in a series of 18 ACCs by array CGH analysis and correlated these with clinicopathologic tumor characteristics and patient survival data.

MATERIALS AND METHODS

Materials and patient data

Eighteen cases of formalin-fixed, paraffin-embedded primary ACCs were included in this study. All tumor samples were obtained from the archives of the Department of Pathology of the VU University Medical Center (Amsterdam, the Netherlands).

Patient follow-up data were available for all 18 cases. Average patient age was 51 years (range, 24-81). ACCs were localized in the major salivary glands (parotid, $n=8$; submandibular, $n=8$; and sublingual, $n=1$) and in the oral cavity ($n=1$). The male/female ratio was 1:2 and the mean follow-up time was 72 months (range, 7-128). Therapy consisted of surgical excision followed by radiotherapy. Eight (45%) patients developed a recurrence and/or a distant metastasis, of which five (62.5%) tumors metastasized to the lungs, one (12.5%) tumor spread to the liver, one to both the liver and the lungs, and one ACC metastasized to the liver, the lungs, and the bones. Development of either recurrence or metastasis was independent of the radicality of the surgical resection margins. Six (33%) patients died of the disease during the time of follow-up.

DNA isolation

Each paraffin block was reviewed to assure that at least 70% tumor cells were present before sectioning and DNA isolation. Genomic DNA was extracted from three 10-µm-thick formalin-fixed, paraffin-embedded tissue sections per case. After deparaffinating with xylene and rehydrating with decreasing concentrations of ethanol (100% - 96% - 70%), the sections were hematoxylin stained and the tumor areas were macrodissected with a surgical blade. This material was collected in a 0.2 ml PCR tube (Greiner) and DNA was isolated using the QIAamp microkit according to the manufacturer's protocols (Qiagen).

Array CGH

CGH BAC microarrays were produced at the Microarray facility of the VU University Medical Center[a] using an OmniGrid 100 microarrayer (Genomic Solutions)[25] and included a total of 4,202 BAC clones with known chromosomal location.[26] Clones selected encompassed clones from the 1 Mb Sanger BAC clone set with an average resolution along the whole genome of 1.0 Mb,[b] the OncoBac set,[c] containing ~600 clones corresponding to 200 cancer-related genes, and selected clones of interest obtained from the Children's Hospital Oakland Research Institute to fill gaps >1 Mb on chromosome 6 and full-coverage contigs of regions on chromosomes 8, 11, 13, and 20.

Hybridizations were essentially performed according to Snijders *et al.*[27] Prehybridization, hybridization, and washing were all done in a hybridization station (HybStation12 – Perkin Elmer Life Sciences).

CGH microarray slides were scanned using a DNA microarray scanner (Agilent Technologies). Spots were quantified using Imagene 5.6 standard edition software (Biodiscovery Ltd.). Spots with nonhomogenous fluorescence were automatically and manually flagged and excluded from the analysis. Subtraction of local background was done for the signal median intensities of both test and reference DNAs.

For the array analysis, BAC clones were positioned along the genome using the May 2004 freeze of the University of California Santa

[a] http://www.vumc.nl/microarrays/
[b] http://www.ensembl.org/Homo_sapiens/cytoview/
[c] http://informa.bio.caltech.edu/Bac_onc.html

Cruz database.[d] For each clone, the average of the triplicate spots was calculated and ratios were normalized by subtraction of the mode value of all BAC and PAC clones on chromosomes 1 to 22. Single clones or triplicate clones with a SD >0.15 were excluded from further analysis. To determine exact breakpoints in the generated array CGH profiles, we segmented the obtained log 2 ratios (tumor signal divided by normal reference signal) by aCGHsmooth.[28] Except in two cases, where thresholds were set at 0.175 and -0.175, smoothed $\log_2$ ratios of ≥ 0.2 were considered a gain, whereas smoothed log 2 ratios of ≤ -0.2 were considered a loss. Steep copy number changes within the graph showing a peak rather than a plateau, with a minimal smoothed $\log_2$ transformed fluorescence ratio of 1.0 or higher, were classified as amplifications, provided that aberrations consisted of at least 3 consecutive BAC clones. The sex chromosomes were discarded from the analysis because all tumor samples were hybridized to reference DNA of the opposite gender.

Statistical analysis

For comparing means of continuous variables between two groups, the nonparametric Mann-Whitney *U* test was used. For testing significance of differences in distribution of categorical variables, the X^2 test was used. Univariate survival analysis was carried out by Kaplan-Meier survival analysis and log-rank testing. *P* values < 0.05 were considered significant. All statistical analyses were carried out with SPSS software version 12.0.1 (SPSS Inc.).

Only clones with <20% missing values were included in the downstream analysis. Smoothed log 2 ratios were manually converted to categorized data (i.e. losses, normals, and gains). To reduce the dimension of the data set, regions of DNA copy number changes were constructed by an algorithm called "CGHregions"[e],[29] implemented using the statistical software environment R (R Development Core Team (2006)). In our settings, we accepted maximally 1% information loss (T=0.01). To identify differential chromosomal loci between two groups of tumors, a Wilcoxon two-sample test corrected for ties was used.[30]

[d] www.genome.ucsc.edu
[e] http://www.few.vu.nl/~mavdwiel/CGHregions.html

This statistical test also includes a false discovery rate correction for multiple testing, needed to discriminate real differences from chance effects. Two-sided false discovery rate values of <0.2 were considered statistically significant, meaning that maximally one of five claimed discoveries is expected to be false.[31]

Results

Most commonly gained regions in ACC harbor growth factors and their receptors

Chromosomal aberrations were observed in all primary ACCs analyzed (n=18), with a total number of 238 chromosomal events. The median amount of copy number changes (gains and losses) per case was 11 (range, 1-36). The frequency plot of alterations per clone of chromosomes 1 to 22 is shown in Figure 1. Significantly more gains than losses were detected in these tumors (205 versus 33), and amplifications were uncommon (n=7; Table 1). The median number of gains was 10 (range, 0-32) and losses was 1.5 (range, 0-5), respectively.

By aligning all carcinoma samples, the smallest regions of overlap (SROs) within the detected chromosomal aberrations were detected. Candidate oncogenes and tumor suppressor genes in these regions were identified by consulting the database of Entrez Gene at the National Center for Biotechnology.[f] Table 2 shows the most frequent SROs together with candidate oncogenes and tumor suppressor genes whose locations coincided with the altered regions. Growth factors and growth factor receptors, which are overexpressed in various tumors, were found in the commonly gained regions.

[f] www.ncbi.nlm.nih.gov/

Together with an increase in DNA copy number at the loci of several fibroblast growth factors (FGF3, FGF4, and FGF19 at 11q13.3 and FGF22 at 19p13.3), a gain was detected at a region containing a FGF receptor (FGFR3 at 4p16.3). Not only the entire long arm of chromosome 22 containing the platelet-derived growth factor (PDGF at 22q13.1) was gained in 67% of our ACCs, but 40% of the tumors also displayed an increase in the DNA amount at the chromosomal area harboring its receptor, PDGFRβ (5q33.1). However, because one tumor only gained a 1.2 Mbp region at 22q13.33 instead of the whole chromosomal arm, the size of this alteration was rate limiting and the PDGF locus was not included in the SROs. Moreover, frequent gains and even amplifications occurred at regions harboring genes involved in signal transduction [e.g., MAPK12 (22q13.3), TRAF2 (9q34.3), and NOTCH1 (9q34.3)] and transcription factors, such as SOX8 (16p13.3), FOSB (19q13.3), and BCL3 (19q13). Chromosomal losses were a less consistent finding than gains, accounting for a maximum of 22% of the tumors (n=4).

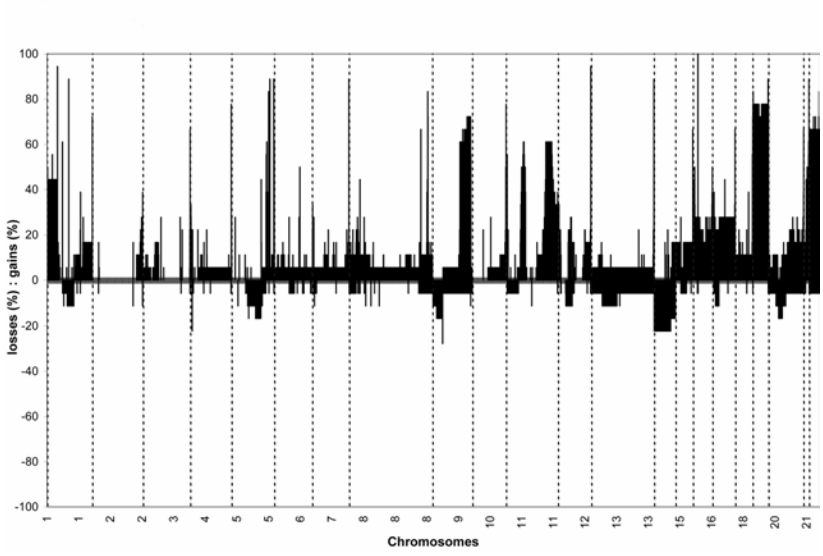

Figure 1 Frequency plots of gains and losses for chromosomes 1 to 22 as determined by array CGH. Percentages of gains (*positive axis*) and losses (*negative axis*) are shown for each BAC clone in all ACCs analyzed.

Cytoband	Amplicon size (Mbps)	Candidate genes
9q34.11	2.0	*PPRX2*
9q34.3	2.9	*TRAF2, NOTCH1,*
11q13.2-q13.3	1.2	*Cyclin D1*
12q14.3-q21.1	6.8	*MDM2, RAP1B, PTPRB*
16p13.3	3.2	*SOX8, MMPL1*
16q24.3	1.5	*CDK10, CDH15, FANCA*
19q13.31-q13.32	0.9	*FOSB*

Table 1 Overview of the amplifications detected in 18 primary ACCs. The amplicon sizes in mega-bps (Mbps) and the candidate oncogenes that are found in the region are also depicted

Correlations of copy number changes with patient data and survival

All array CGH data were correlated with histologic type (cribriform/tubular or solid), gender (male or female), and clinical follow-up data. The presence of gains of the regions 5q35.1-q35.3 (P=0.03), 7p22.3-p22.1 (P=0.05), and of 16q24.3 (P=0.006) together with a loss at region 14q11.2-q31.3 (P=0.05) was significantly correlated with the solid subtype when compared to the cribriform/tubular type. Gain of the region 14q32.12-q32.33 (P=0.02) was significantly more frequent in men. Interestingly, univariate analysis showed that patients with 17 or more aberrations exhibited a significantly shorter survival time than patients with less chromosomal events (log rank=5.2; P=0.02; Figure 2). Correlation of the amount of aberrations with clinicopathologic parameters showed a tendency toward more chromosomal losses in tumors that recurred or metastasized during the time of follow-up (P=0.08). Gender and histologic subtypes (cribriform/tubular versus solid) did not correlate with the number of chromosomal aberrations, although the solid subtype tended to have more chromosomal losses when compared with the other histologic group (P=0.06).

	Cytoband	Size of region (Mbps)	No. tumors *n* (%)	Candidate genes
Gains	1p36.33-p35.3	27.70	8 (44)	*E2F2, TNFRSF4*
	1p35.2-p33	16.11	8 (44)	*CSF3R, PTPRF, HDAC1*
	2q37.3	0.58	5 (28)	-
	4p16.3	1.69	5 (28)	*FGFR3, CTBP1*
	5q32-q33.1	3.67	7 (39)	*PDGFRB*
	8q24.3	3.80	7 (39)	*PLEC1*
	9q33.3-q34.3	11.44	13 (72)	*ABL1, NOTCH1, TRAF2*
	11p15.5	1.82	6 (33)	*MUC2, HRAS*
	11q13.3	0.37	11 (61)	*FGF3, FGF4, FGF19*
	11q23.3	2.30	11 (61)	*IL10RA, MLL, CBL*
	12q13.2-q14.1	1.77	5 (28)	*ERBB3, STAT6, RAB5B,WNT1*
	13q34	1.04	6 (33)	*TFDP1, GAS6*
	16p13.3	4.42	8 (44)	*MMP25,SOX8,RAB26*
	16q24.3	0.05	7 (39)	-
	17p13.3	1.84	7 (39)	*CRK*
	17q11.2-q25.3	55.15	5 (28)	*ERBB2, PPMD1, ITGB4*
	19p13.3-p13.11	17.90	14 (78)	*RAB8A, FGF22, ICAM5*
	19q12-q13.43	26.75	14 (78)	*TGFB1, BCL3*
	20q13.33	2.64	5 (28)	*SOX18, PTK6,RTEL1,*
	21q22.3	3.21	11 (61)	*ITGB2, PTTG1IP,*
	22q13.33	1.21	13 (72)	*ECGF1, MAPK12*
Losses	1p21.3-p12	23.73	2 (11)	*ST7L*
	5q21.1	2.49	3 (17)	*ST8SIA4*
	5q21.3-q23.2	18.82	3 (17)	*APC*
	9p22.3-p13.3	20.47	3 (17)	*CDKN2A*
	12q12-q14.1	23.01	2 (11)	*KRT7, HOXC5*
	13q14.3-q21.32	13.22	2 (11)	*PCDH17, PCDH9, INTS6*
	14q11.2-q31.3	64.52	4 (22)	*SEL1, DLG7, NFKBIA*
	17p13.1-q11.1	12.93	2 (11)	*LLGL1*
	20q11.23-q12	1.98	3 (17)	*DHX35*

Table 2 Most frequently gained (>25%) and lost (>10%) minimal common regions of overlap were detected by array CGH in 18 primary ACCs, together with candidate oncogenes and tumor suppressor genes. The size of the aberration is depicted in mega-bps (Mbps).

Cytoband	Size (Mbps)	Bp position start	Bp position end	No. clones	P	FDR value
4p13.3	1.09	1764749	2859130	3	0.03	0.15
11q23.3-q24.1	0.62	120648888	121267168	6	0.05	0.20
16p13.3	0.78	3299612	4084406	3	0.05	0.20
16q24.1-q24.2	3.73	83444369	87172104	4	0.03	0.15
17p13.1	0.89	6894326	7781828	6	0.03	0.15

Table 3 Chromosomal regions analyzed by array CGH with significantly different alterations between tumors with metastasis/recurrence during follow-up and those without. Sizes of the regions are depicted in mega-basepares (Mbps), together with start and end sequences of the BAC clones in bp. Regions consisting of less than three clones were not considered reliable. Abbreviation: FDR, false discovery rate.

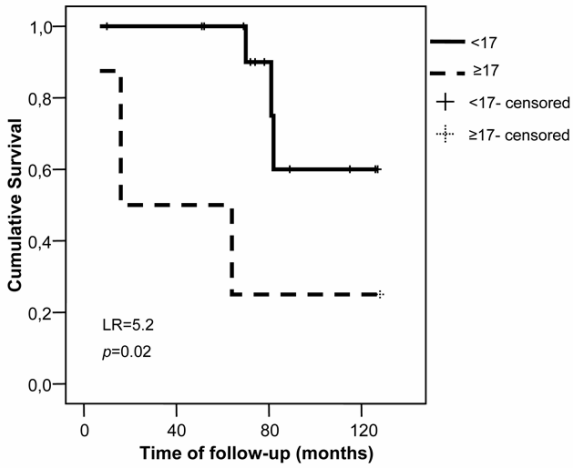

Figure 2 Kaplan-Meier plot for overall survival in primary ACCs. Patients with 17 or more chromosomal events in their tumors show a significantly poorer survival (P=0.02) than patients with less chromosomal aberrations.

Five chromosomal regions correlate with recurrence/metastasis

To identify genomic alterations, which may contribute to aggressive biological behavior of ACC, we analyzed the chromosomal regions displaying significant differences between ACCs that had developed a recurrence/metastasis during the time of follow-up (n=9; mean follow-up, 59 months) and those that had not (n=9; mean follow-up, 87 months).

Because distant metastases occur in ~50% of ACC patients regardless of local tumor control,[32] it seems that two distinct biological pathways are responsible for the occurrence of locoregional recurrence and metastasis. However, as mentioned before, development of either a recurrence or metastasis in our group of tumors was independent of the radicality of the surgical margins of the resection. Dividing our tumors according to clinical outcome would have hampered reliable statistical results due to our relatively small sample size. Therefore, it was decided to regard the presence of either a recurrence or metastasis as a measure for aggressive ACC behavior and to analyze our results according to this criterion.

Using the Wilcoxon, two-sample test corrected for ties with false discovery rate correction, five significantly differential genomic alterations were found on the chromosomal loci 4p16.3, 11q23.3-q24.1, 16p13.3, 16q24.1-q24.2, and 17p13.1. Except for the area on chromosome 11, the gains on chromosomes 4, 16, and 17 were found significantly more frequently gained in tumors that recurred/metastasized during the time of follow-up (Table 3).

Aggressive ACC behavior is characterized by a differential increase in DNA copy numbers at the chromosomal areas 4p16.3, 16p13.3, 16q24.1-q24.2, and 17p13.1. Interestingly, the genes *FGFR3* and *FGF11* are located at these altered loci (i.e., at 4p16.3 and 17p13.1, respectively). Other genes with a possible role in ACC progression are the *CREB binding protein* (*CREBBP*) and the *TNF receptor-associated protein 1* (*TRAP1*) at 16p13.3 and the transcription factor *Forkhead box C2* (*FOXC2*) at 16q24.1-q24.2.

Tumors exhibiting indolent biological behavior during the time of follow-up showed more frequently a copy number gain on chromosome 11. The only one gene that is located in this 0.62 Mbp area, the *sortilin-*

related receptor containing LDLR class gene (*SORL1*), might be involved in preventing ACC cell proliferation and invasion.

DISCUSSION

For ACCs, current prognosis depends on clinicopathologic variables, such as tumor-node-metastasis stage, tumor grade, and histologic patterns.[33] However, these parameters do not uniformly predict tumor behavior, necessitating the identification of novel prognostic markers.

The first aim of this study was to uncover commonly altered chromosomal regions in a relatively large group of ACCs with a higher resolution than has been achieved until now. More insight in common genomic alterations is an essential first step not only to uncover genes involved in ACC initiation but also to identify biomarkers for a better risk stratification of patients. Furthermore, genetic changes that lead to the progression of ACC are poorly characterized. Despite resection with or without postoperative irradiation, ~40% to 60% of patients with salivary ACC develop distant metastases, which are the main cause of disease-specific mortality.[34] It is therefore important to characterize chromosomal regions associated with invasive tumor behavior leading to poor patient outcome. As a second aim, individual clones that were significantly different between tumors with recurrence/metastasis during the time of follow-up and those without were distinguished. These differential genomic alterations might reveal genes responsible for aggressive ACC behavior.

The ACCs in our series displayed a median number of 11 aberrations per tumor. We found significantly more gains than losses in our cohort of neoplasms (205 versus 33, respectively), which agrees with earlier studies.[19,21] Unlike in cervical carcinomas,[35] amplifications are not a frequent event ($n=7$) in our group of tumors.

Previously reported and frequently changed loci in ACC include gains on 16p, 17q, 22q13[20] and losses on 6q[6,20,36] and 12q12-q13.[22] Translocations seem to be conserved to chromosomes 6, 9, and 12.[37,38] With the exception of a recent study by Kasamatsu *et al.*,[19] all previous cytogenetic analyses on ACCs report of a relatively limited number of chromosomal aberrations. The high number of alterations in our study

could be explained first by the very high-resolution quantitative detection of copy number changes by array CGH in contrast to LOH or classical CGH and second by the relatively large amount of samples in our group of tumors.

In addition to the most commonly observed gains in ACCs previously described by others, we detected novel copy number changes at a high frequency in our group of tumors. Gains of the regions 9q33.3-q34.3, 11q13.3, 11q23.3, 19p13.3-p13.11, 19q12-q13.43, and 21q22.3 were present in ~60 to 80% of ACCs tested, suggesting that these loci in particular harbor oncogenes essential for ACC initiation.

The most frequently detected LOH in salivary gland neoplasms is at locus 6q23-q25.[2,4,6] Although LOH is not detectable by array CGH, we observed one tumor in our group exhibiting a loss in the same chromosomal region. A frequent loss observed at 12q12-q13 in 33% of ACCs by El-Rifai *et al*.[22,39] has also been identified in our group of tumors, although at a lower frequency (11%). LOH rates in ACCs were also shown to correlate with unfavorable disease course, higher grade, and the presence of the solid histologic pattern.[2,40] Our tumors displayed a tendency toward more chromosomal losses in the solid subtype (P=0.06).

Our results are in concordance with earlier observations by Freier *et al*., where a copy number gain of 22q13 was found to be the most consistent aberration in 27 ACCs.[20] Although our smallest region of overlap on this chromosome maps to the region 22q13.33 in 72% of the ACCs, the complete long arm of chromosome 22, which is also gained in certain sarcomas,[41] was gained in 68% of our tumors. PDGF at 22q13.1 is a mesenchymal cell mitogen and operates in connective tissue growth, wound healing, and angiogenesis. Expression of PDGF was shown to correlate with advanced tumor stages and poor survival in mamma carcinomas.[42] Moreover, a metastasis-specific gene set in breast tumors not only included PDGF and components of its signaling pathway but also suggested the existence of an autocrine signaling loop, which was thought to occur almost exclusively in nonepithelial tumors.[43] A previous gene expression analysis detected the PDGFβ receptor to be overexpressed in advanced stages of prostate cancer and to predict recurrence.[44] Interestingly, DNA copy numbers at the locus of the PDGFRβ on chromosome 5 are increased in ~40% of our ACCs. Thus, an

autocrine PDGF/PDGFR loop can be established through the possible up-regulation of both ligand and receptor contributing to ACC tumorigenesis.

Our finding that 61% of our tumors display a DNA copy number increase correlating with loci for *FGF(R)*s suggests an important role for this family of mitogens in ACC development. Furthermore, association of genomic alterations with aggressive tumor behavior detected more frequently a gain at the regions for *FGF11* (17p13.1) and *FGFR3* (4p16.3) in recurred/metastasized ACCs. This finding suggests that these growth factors are not only involved in ACC initiation but also seem to be associated with its aggressive behavior. Several lines of evidence suggest a function for FGF signaling in salivary gland progression. Immunohistochemically, FGF1, FGF2, and FGFR1 have already been detected to be overexpressed in human salivary gland tumors and implicated in their carcinogenesis.[45,46] More specifically, experiments in a human salivary gland adenocarcinoma cell line showed that during the process of malignant transformation, *de novo* expression of FGFR1 and FGFR4 coincides with the down-regulation of the keratinocyte growth factor receptor. Transfection of wild-type keratinocyte growth factor receptor induced differentiation and apoptosis together with suppression of tumor cell growth *in vitro* and *in vivo*.[47] Overexpression of FGFs and their receptors has also been reported in several other malignancies.[48] For example, the most common genetic alterations contributing to the initiation of multiple myeloma or urothelial cell carcinoma are considered activating mutations in distinct exons or translocations, which cause FGFR3 to be overexpressed. The presence of FGFs together with an FGFR in commonly gained regions may imply the existence of a second autocrine stimulation loop in ACCs promoting tumor progression.

The chromosomal region 11q23.3-q24.1 was significantly more frequently gained in indolent tumors compared with recurred/metastasized ACCs. The only gene located at this locus, *SORL1*, might thus be involved in the reduction of cell proliferation and prevention of cell invasion. SORL1 belongs to the family of lipoprotein receptor proteins and is a multifunctional endocytic receptor that may be implicated in the uptake of lipoproteins and of proteases. Furthermore, this protein also might play a role in cell-cell adhesion.

Interestingly, an *in vitro* experiment on mouse Swiss 3T3 and rat lung fibroblasts showed that PDGF-stimulated cell proliferation is negatively influenced by lipoprotein receptor proteins.[49] A lipoprotein receptor protein-dependent mechanism seems to clear away the PDGF-BB-α2M-plasmin complex and thus inhibiting its growth stimulating effect. We hypothesize that a similar mechanism involving overexpressed SORL1 could be responsible for the prevention of PDGF driven cell proliferation and invasion in ACCs.

In summary, we found several chromosomal regions in our 18 ACCs, which were gained at a high frequency (60-80%), suggesting that these particular areas harbor potential oncogenes important for ACC initiation. Interestingly, several loci coincide with growth factors, such as *FGFs* and *PDGF*, and their receptors, suggesting a role for autocrine stimulation in ACC tumorigenesis. Knocking out the expression of FGFs and PDGFs or interfering with their receptors in a model system and evaluating the effect of this intervention on the tumorigenic phenotype could validate their involvement in ACC development.

This study has significantly expanded the spectrum of previously found aberrations in ACCs. However, further evaluation and fine mapping of the differentially and frequently gained chromosomal regions is necessary to determine their role in the initiation and progression of ACC.

Acknowledgments

We thank the mapping core and map finishing groups of the Wellcome Trust Sanger Institute for initial BAC clone supply and verification and M. Tijssen, P.P. Eijk, E. Hopmans, and P. van den IJssel for printing slides and technical assistance.

REFERENCES

1. Spiro RH, Huvos AG, and Strong EW. Adenoid cystic carcinoma of salivary origin. A clinicopathologic study of 242 cases. Am J Surg 1974, 128: 512-520.
2. Stallmach I, Zenklusen P, Komminoth P, Schmid S, Perren A, Roos M, Jianming Z, Heitz PU, and Pfaltz M. Loss of heterozygosity at chromosome 6q23-25 correlates with clinical and histologic parameters in salivary gland adenoid cystic carcinoma. Virchows Arch 2002, 440: 77-84.
3. Roijer E, Dahlenfors R, Mark J, and Stenman G. Observations by chromosome banding, FISH and immunohistochemistry in an adenoid cystic carcinoma with del(17)(p13) as the sole deviation. Virchows Arch 1997, 430: 339-342.
4. Nordkvist A, Mark J, Gustafsson H, Bang G, and Stenman G. Non-random chromosome rearrangements in adenoid cystic carcinoma of the salivary glands. Genes Chromosomes Cancer 1994, 10: 115-121.
5. Jin C, Martins C, Jin Y, Wiegant J, Wennerberg J, Dictor M, Gisselsson D, Strombeck B, Fonseca I, Mitelman F, Tanke HJ, Hoglund M, and Mertens F. Characterization of chromosome aberrations in salivary gland tumors by FISH, including multicolor COBRA-FISH. Genes Chromosomes Cancer 2001, 30: 161-167.
6. Queimado L, Reis A, Fonseca I, Martins C, Lovett M, Soares J, and Parreira L. A refined localization of two deleted regions in chromosome 6q associated with salivary gland carcinomas. Oncogene 1998, 16: 83-88.
7. Li X, Tsuji T, Wen S, Mimura Y, Wang Z, Sasaki K, and Shinozaki F. A fluorescence in situ hybridization (FISH) analysis with centromere-specific DNA probes of chromosomes 3 and 17 in pleomorphic adenomas and adenoid cystic carcinomas. J Oral Pathol Med 1995, 24: 398-401.
8. Chandan VS, Wilbur D, Faquin WC, and Khurana KK. Is c-kit (CD117) immunolocalization in cell block preparations useful in the differentiation of adenoid cystic carcinoma from pleomorphic adenoma? Cancer 2004, 102: 207-209.
9. Dori S, Trougouboff P, David R, and Buchner A. Immunohistochemical evaluation of estrogen and progesterone receptors in adenoid cystic carcinoma of salivary gland origin. Oral Oncol 2000, 36: 450-453.
10. Vered M, Braunstein E, and Buchner A. Immunohistochemical study of epidermal growth factor receptor in adenoid cystic carcinoma of salivary gland origin. Head Neck 2002, 24: 632-636.
11. Cho KJ, Lee SS, and Lee YS. Proliferating cell nuclear antigen and c-erbB-2 oncoprotein expression in adenoid cystic carcinomas of the salivary glands. Head Neck 1999, 21: 414-419.
12. Shintani S, Funayama T, Yoshihama Y, Alcalde RE, Ootsuki K, Terakado N, and Matsumura T. Expression of c-erbB family gene products in adenoid cystic carcinoma of salivary glands: an immunohistochemical study. Anticancer Res 1995, 15: 2623-2626.
13. Yamamoto Y, Wistuba II, Kishimoto Y, Virmani AK, Vuitch F, Albores-Saavedra J, and Gazdar AF. DNA analysis at p53 locus in adenoid cystic carcinoma: comparison of molecular study and p53 immunostaining. Pathol Int 1998, 48: 273-280.
14. Zhu QR, White FH, and Tipoe GL. p53 oncoprotein accumulation in adenoid cystic carcinoma of parotid and palatine salivary glands. Pathology 1997, 29: 154-158.
15. Yamamoto Y, Virmani AK, Wistuba II, McIntire D, Vuitch F, Albores-Saavedra J, and Gazdar AF. Loss of heterozygosity and microsatellite alterations in p53 and RB genes in adenoid cystic carcinoma of the salivary glands. Hum Pathol 1996, 27: 1204-1210.
16. Frierson HF, Jr., El Naggar AK, Welsh JB, Sapinoso LM, Su AI, Cheng J, Saku T, Moskaluk CA, and Hampton GM. Large scale molecular analysis identifies genes with

altered expression in salivary adenoid cystic carcinoma. Am J Pathol 2002, 161: 1315-1323.
17. Maruya S, Kim HW, Weber RS, Lee JJ, Kies M, Luna MA, Batsakis JG, and El Naggar AK. Gene expression screening of salivary gland neoplasms: molecular markers of potential histogenetic and clinical significance. J Mol Diagn 2004, 6: 180-190.
18. Oostlander AE, Meijer GA, and Ylstra B. Microarray-based comparative genomic hybridization and its applications in human genetics. Clin Genet 2004, 66: 488-495.
19. Kasamatsu A, Endo Y, Uzawa K, Nakashima D, Koike H, Hashitani S, Numata T, Urade M, and Tanzawa H. Identification of candidate genes associated with salivary adenoid cystic carcinomas using combined comparative genomic hybridization and oligonucleotide microarray analyses. Int J Biochem Cell Biol 2005, 37: 1869-1880.
20. Freier K, Flechtenmacher C, Walch A, Ohl S, Devens F, Burke B, Hassfeld S, Lichter P, Joos S, and Hofele C. Copy number gains on 22q13 in adenoid cystic carcinoma of the salivary gland revealed by comparative genomic hybridization and tissue microarray analysis. Cancer Genet Cytogenet 2005, 159: 89-95.
21. Toida M, Balazs M, Mori T, Ishimaru JI, Ichihara H, Fujitsuka H, Hyodo I, Yokoyama K, Tatematsu N, and Adany R. Analysis of genetic alterations in salivary gland tumors by comparative genomic hybridization. Cancer Genet Cytogenet 2001, 127: 34-37.
22. El Rifai W, Rutherford S, Knuutila S, Frierson HF, Jr., and Moskaluk CA. Novel DNA copy number losses in chromosome 12q12--q13 in adenoid cystic carcinoma. Neoplasia 2001, 3: 173-178.
23. De Vries S, Nyante S, Korkola J, Segraves R, Nakao K, Moore D, Bae H, Wilhelm M, Hwang S, and Waldman F. Array-based comparative genomic hybridization from formalin-fixed, paraffin-embedded breast tumors. J Mol Diagn 2005, 7: 65-71.
24. Pinkel D and Albertson DG. Array comparative genomic hybridization and its applications in cancer. Nat Genet 2005, 37 Suppl: S11-S17.
25. Van den Ijssel P, Tijssen M, Chin SF, Eijk P, Carvalho B, Hopmans E, Holstege H, Bangarusamy DK, Jonkers J, Meijer GA, Caldas C, and Ylstra B. Human and mouse oligonucleotide-based array CGH. Nucleic Acids Res 2005, 33: e192.
26. Schreurs MW, Hermsen MA, Geltink RI, Scholten KB, Brink AA, Kueter EW, Tijssen M, Meijer CJ, Ylstra B, Meijer GA, and Hooijberg E. Genomic stability and functional activity may be lost in telomerase-transduced human CD8+ T lymphocytes. Blood 2005, 106: 2663-2670.
27. Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Huey B, Kimura K, Law S, Myambo K, Palmer J, Ylstra B, Yue JP, Gray JW, Jain AN, Pinkel D, and Albertson DG. Assembly of microarrays for genome-wide measurement of DNA copy number. Nat Genet 2001, 29: 263-264.
28. Jong K, Marchiori E, Meijer G, Vaart AV, and Ylstra B. Breakpoint identification and smoothing of array comparative genomic hybridization data. Bioinformatics 2004, 20: 3636-3637.
29. Van de Wiel MA and Van Wieringen WN. CGHregions: Dimension reduction for Array CGH data with minimal information loss. Cancer Informatics 2007, 2: 55-63.
30. Van de Wiel MA, Smeets SJ, Brakenhoff RH, and Ylstra B. CGHMultiArray: exact p-values for multi-array CGH-data. Bioinformatics 2005, 21: 3193-3194.
31. Dupuy A and Simon RM. Critical review of published microarray studies for cancer outcome and guidelines on statistical analysis and reporting. J Natl Cancer Inst 2007, 99: 147-157.
32. Matsuba HM, Thawley SE, Simpson JR, Levine LA, and Mauney M. Adenoid cystic carcinoma of major and minor salivary gland origin. Laryngoscope 1984, 94: 1316-1318.
33. Lima RA, Tavares MR, Dias FL, Kligerman J, Nascimento MF, Barbosa MM, Cernea CR, Soares JR, Santos IC, and Salviano S. Clinical prognostic factors in malignant parotid gland tumors. Otolaryngol Head Neck Surg 2005, 133: 702-708.

34. Spiro RH. Distant metastasis in adenoid cystic carcinoma of salivary origin. Am J Surg 1997, 174: 495-498.
35. Rao PH, Arias-Pulido H, Lu XY, Harris CP, Vargas H, Zhang FF, Narayan G, Schneider A, Terry MB, and Murty VV. Chromosomal amplifications, 3q gain and deletions of 2q33-q37 are the frequent genetic changes in cervical carcinoma. BMC Cancer 2004, 4: 5.
36. Rutherford S, Yu Y, Rumpel CA, Frierson HF, Jr., and Moskaluk CA. Chromosome 6 deletion and candidate tumor suppressor genes in adenoid cystic carcinoma. Cancer Lett 2006, 236: 309-317.
37. Martins C, Fonseca I, Roque L, Pinto AE, and Soares J. Malignant salivary gland neoplasms: a cytogenetic study of 19 cases. Eur J Cancer B Oral Oncol 1996, 32B: 128-132.
38. Martins C, Fonseca I, Roque L, Ribeiro C, and Soares J. Cytogenetic similarities between two types of salivary gland carcinomas: adenoid cystic carcinoma and polymorphous low-grade adenocarcinoma. Cancer Genet Cytogenet 2001, 128: 130-136.
39. Rutherford S, Hampton GM, Frierson HF, and Moskaluk CA. Mapping of candidate tumor suppressor genes on chromosome 12 in adenoid cystic carcinoma. Lab Invest 2005, 85: 1076-1085.
40. El Naggar AK, Hurr K, Kagan J, Gillenwater A, Callender D, Luna MA, and Batsakis JG. Genotypic alterations in benign and malignant salivary gland tumors: histogenetic and clinical implications. Am J Surg Pathol 1997, 21: 691-697.
41. Lee MW, Jee KJ, Han SS, Gong GY, Choi JH, Moon KC, and Koh JK: Comparative genomic hybridization in epithelioid sarcoma. Br J Dermatol 2004, 151: 1054-1059
42. Ariad S, Seymour L, and Bezwoda WR. Platelet-derived growth factor (PDGF) in plasma of breast cancer patients: correlation with stage and rate of progression. Breast Cancer Res Treat 1991, 20: 11-17.
43. Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodiecci P, Donovan M, Cordon-Cardo C, Beug H, and Grunert S. Autocrine PDGFR signaling promotes mammary cancer metastasis. J Clin Invest 2006, 116: 1561-1570.
44. Singh D, Febbo PG, Ross K, Jackson DG, Manola J, Ladd C, Tamayo P, Renshaw AA, D'Amico AV, Richie JP, Lander ES, Loda M, Kantoff PW, Golub TR, and Sellers WR. Gene expression correlates of clinical prostate cancer behavior. Cancer Cell 2002, 1: 203-209.
45. Myoken Y, Myoken Y, Okamoto T, Sato JD, Kan M, McKeehan WL, Fujihara M, and Takada K. Immunohistochemical localization of fibroblast growth factor-1 (FGF-1), FGF-2 and fibroblast growth factor receptor-1 (FGFR-1) in pleomorphic adenoma of the salivary glands. J Oral Pathol Med 1997, 26: 17-22.
46. Myoken Y, Myoken Y, Okamoto T, Sato JD, Kan M, McKeehan WL, Nakahara M, and Takada K. Immunohistochemical study of overexpression of fibroblast growth factor-1 (FGF-1), FGF-2, and FGF receptor-1 in human malignant salivary gland tumours. J Pathol 1996, 178: 429-436.
47. Zhang Y, Wang H, Toratani S, Sato JD, Kan M, McKeehan WL, and Okamoto T. Growth inhibition by keratinocyte growth factor receptor of human salivary adenocarcinoma cells through induction of differentiation and apoptosis. Proc Natl Acad Sci U S A 2001, 98: 11336-11340.
48. Grose R and Dickson C. Fibroblast growth factor signaling in tumorigenesis. Cytokine Growth Factor Rev 2005, 16: 179-186.
49. Bonner JC, Badgett A, Hoffman M, and Lindroos PM. Inhibition of platelet-derived growth factor-BB-induced fibroblast proliferation by plasmin-activated alpha 2-macroglobulin is mediated via an alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein-dependent mechanism. J Biol Chem 1995, 270: 6389-6395.

CHAPTER 3

High Expression of Polycomb Group Protein EZH2 Predicts Poor Survival in Salivary Gland Adenoid Cystic Carcinoma

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Journal of Clinical Pathology 2008; 61(6): 744-749.

Chapter 3

ABSTRACT

The prognosis of adenoid cystic carcinoma (ACC), a malignant salivary gland tumor, depends on clinicopathological parameters. To decipher the biological behavior of ACC, and to identify patients at risk of developing metastases, additional markers are needed. Expression of the cell cycle proteins p53, Cyclin D1, $p16^{INK4a}$, E2F1 and Ki-67, together with the Polycomb group (PcG) proteins BMI-1, MEL-18, EZH2, and EED was investigated immunohistochemically in 21 formalin-fixed, paraffin-embedded primary ACCs in relation to tumor characteristics. ACC revealed significantly increased expression of the cell cycle proteins compared to normal salivary tissue (n=17). Members of the two PcG complexes displayed mutually exclusive expression in normal salivary gland tissue, with BMI-1 and MEL-18 being abundantly present. In ACC, this expression pattern was disturbed, with EZH2 and EED showing significantly increased expression levels. In univariate analysis, presence of recurrence, poor differentiation and high EZH2 levels (>25% immunopositivity) significantly correlated with unfavorable outcome. ACCs with high proliferative rate (>25% Ki-67 immunopositivity) significantly correlated with high levels of EZH2 and p16. Only the development of recurrence was an independent prognostic factor of survival in multivariate analysis. In conclusion, expression of PcG complexes and of essential cell cycle proteins is highly deregulated in ACC. Also, EZH2 expression has prognostic relevance in this malignancy.

INTRODUCTION

Adenoid cystic carcinoma (ACC) is a relatively common malignant salivary gland tumor, representing about 10-15% of all head and neck neoplasms.[1] This tumor shows bidirectional differentiation towards luminal (ductal) and abluminal (myoepithelial and basal) cells, in which the myoepithelial cells predominate.[2] Histologically, three distinct patterns are recognized, namely the cribriform, tubular and solid subtype.[3]

Given that this neoplasm is characterized by slow progression, about 65% of the patients are still alive after 5 years. However, local recurrences and late distant metastases result in a 20 year survival rate of only about 20%.[4] To identify patient groups with unfavorable outcome, several studies have attempted to uncover clinical and pathologic parameters with prognostic relevance in ACC.[5-7] However, these clinicopathologic parameters are far from satisfactory as prognostic predictors and several studies have been undertaken to search for additional markers based on protein levels.[8-13] Only p53 proved to be a consistent marker of aggressiveness, being highly expressed in the solid pattern,[10] correlating with unfavorable clinical outcome,[14] and having high LOH rates.[15]

Polycomb group (PcG) proteins control the transcriptional memory of a cell by maintaining the stable silencing of specific sets of genes through chromatin modifications.[16] They form two distinct complexes and their members exhibit a mutually exclusive expression pattern in differentiated, healthy tissues.[17] A growing body of work has linked deregulated expression of human PcG genes to malignant transformation, loss of differentiation in tumor cells, metastatic behavior, and poor prognosis.[18, 19] At the molecular level, the link between aberrant PcG gene expression and cancer development remains largely unclear. However, recent in vitro studies have demonstrated that PcG proteins interact with several negative ($p16^{INK4a}$, p53, pRb, $p14^{ARF}$) [20-22] and positive regulators (E2F1, Cyclin D1)[23] of the cell cycle. Disturbances in PcG protein interaction with these cell cycle controllers have been suggested to be an important step in malignant transformation.

The present study was undertaken to evaluate immunohistochemically the expression patterns of various cell cycle-associated proteins ($p16^{INK4a}$, Cyclin D1, p53, E2F1 and Ki-67) and PcG proteins (BMI-1, MEL-18, EZH2, and EED), and to assess their potential prognostic role in predicting tumor characteristics and patient survival in the (myo)epithelial tumor ACC.

MATERIALS AND METHODS

Patient data

Twenty-one patients, from whom formalin-fixed, paraffin-embedded (FFPE) primary ACCs were available, were included in this study. In 17 cases, matched normal salivary gland tissue could be obtained as a control. All tumor samples were derived from the archives of the Department of Pathology of the VU University Medical Center, Amsterdam, the Netherlands. Patients were diagnosed with ACC between 1993 and 1999. The design of this study adheres to the code for proper secondary use of human tissue of the Dutch Federation of Biomedical Scientific Societies (http://www.federa.org).[24] Patient follow-up data were available for all 21 cases. The male to female ratio was 1:1.3 and patients ranged in age from 25 to 81 years (median 54.9). Nineteen tumors were located in the major salivary glands (9 parotid, 8 submandibular, 2 sublingual) and two were located elsewhere in the oral cavity. Tumors were classified by a pathologist (EB) according to the predominant histologic pattern (6 solid, 15 cribriform/tubular). Except for two patients who received surgery alone, all patients were treated by surgery and postoperative radiotherapy. Five patients (24%) developed a recurrence, eight patients (38%) developed a metastasis, and two patients (9%) developed both during the time of follow up (range 7-128; median 73.6). Most ACCs spread to the lungs (n=5), one tumor metastasized to the liver, one to the liver and the lungs and one spread to the liver, lungs and bones. Development of either recurrence or metastasis was independent of the radicality of the surgical resection margins. Disease-free survival ranged from 2 to 121 months (median

27). Six (29%) patients died during the time of follow-up, all due to the disease.

Immunohistochemistry

All tumor samples were fixed in 4% buffered formalin, processed, and embedded in paraffin according to routine procedures. Endogenous peroxidase was inhibited with 0.3% H_2O_2 in methanol at room temperature; antigens were retrieved by heating either in the microwave (antibodies BMI-1 and $p16^{INK4a}$ in Tris/EDTA buffer pH 9; Ki-67 and p53 antibodies in 0.01 M sodium citrate buffer pH 6.0) or in the autoclave (EZH2 in Tris/EDTA buffer pH 8; MEL-18 and Cyclin D1 in 0.01 M sodium citrate buffer pH 6.0). In case of PcG antibodies, the slides were rinsed in phosphate-buffered saline (PBS) containing 0.5% Triton-X (5 minutes), followed by PBS only (3 x 1 min). The slides were washed in 0.1 M Glycine (10 min) and rinsed in PBS. After preincubation with normal rabbit serum (NRS), primary and biotinylated secondary antibodies (rabbit anti-mouse $F(ab')_2$ and rabbit anti-goat together with 5% Human Pool Serum (HPS)) were applied and immunostaining was performed using the appropriate detection method (Table 1). Negative controls were included by substitution of the primary antibody with 1% Bovine Serum Albumin (BSA) in PBS. The streptavidin and biotinylated horseradish peroxidase complex (sABC) method was performed with a biotinylated tyramine (BT) intensification step (1:1000 with 0.01% H_2O_2 in sterile PBS). Envision horseradish peroxidase system (Dako, Glostrup, Denmark) and Powervision Plus (Immunologics, Duiven, The Netherlands) were carried out following the protocol supplied by the manufacturer. 3-amino-9-ethylcarbazole (AEC) (Zymed, San Francisco, CA, USA) was used as substrate for the sABC-BT method, and diaminobenzidine (DAB) for the Envision and Powervision Plus method. Sections were counterstained with haematoxylin, dehydrated and mounted. Interpretation of the staining was done by semi-quantitative scoring by at least two investigators (HV and EB). Percentage of tumor cells with positive staining was grouped as low (expression in ≤25% of cells) or high (expression in >25% of cells).

Antibody	Clone	Source	Species	Dilution	Detection method
P53	D07	DAKO	Mouse	1:500 o/n	sABC-BT
P16	16P04	Neomarkers	Mouse	1:200 1h	Envision
E2F1	KH95	Neomarkers	Mouse	1:250 1 h	Powervision Plus
Ki-67	MIB-1	DAKO	Mouse	1:40 o/n	sABC-BT
Cyclin D1	DCS-6	Neomarkers	Mouse	1:400 o/n	Envision
BMI-1		*	Mouse	1:1000 o/n	sABC-BT
EZH2	M18	*	Mouse	1:5 o/n	sABC-BT
MEL-18	C-20	Santa Cruz	Goat	1:400 o/n	sABC-BT
EED	M26	*	Mouse	1:25 o/n	sABC-BT

Table 1 Antibodies and staining conditions for immunohistochemistry. Abbreviations: o/n, overnight at 4ºC; 1 h, 1 hour at room temperature; sABC-BT, horseradish-peroxidase conjugated streptavidin/biotin complex and biotinylated tyramine; * non-commercial

Statistical analysis

Because of the non-normal distribution of the protein expression levels and the relatively small sample size, statistical evaluation was performed using non-parametric tests. Comparison between the protein expression levels in tumor and normal tissue was done with the use of the Mann Whitney U test. Associations between protein expression and clinicopathological parameters were performed using Fischer's exact probability test. Disease-free and disease-specific survival rates were determined by the Kaplan-Meier method; differences in survival were evaluated with the log-rank test. Univariate analysis with the Cox proportional hazards regression model was used to determine each identified prognostic factor. The multivariate Cox proportional hazards regression model was employed in a forward stepwise manner to analyze the relevance of independent prognostic factors. A two-sided p value of ≤ 0.05 was considered to be statistically significant. Statistical analyses were performed using SPSS, V.12.0.1 (SPSS Inc., Chicago, IL, USA).

RESULTS

Cell cycle protein expression in ACC is disturbed compared to normal salivary gland tissue

Seventeen cases of normal salivary gland tissue were investigated immunohistochemically for the expression of the cell cycle proteins E2F1, Ki-67, $p16^{INK4a}$, p53 and Cyclin D1. All proteins showed low median expression levels (Figure 1a): E2F1, 3% (range 1-5); Ki-67, 3% (range 1-15); $p16^{INK4a}$, 3% (range 1-10); p53, 3% (range 1-10); and Cyclin D1, 1% (range 1-10). In ACC, expression of all the cell cycle proteins was significantly increased compared to normal salivary gland tissue (Figure 1a-d). E2F1 showed a median expression of 20% (range 5-90), Ki-67 of 15% (range 1-60), $p16^{INK4a}$ of 20% (range 1-95), p53 of 65% (range 5-90), and Cyclin D1 of 20% (range 1-75).

Mutually exclusive expression pattern of PcG complexes in normal salivary gland tissue is deregulated in ACC

Members of the PRC1 complex, BMI-1 and MEL-18, showed abundant nuclear staining in normal salivary gland tissue (median expression 95% (range 80-100) and 40% (range 10-95), respectively; Figure 2a). In contrast, members of the PRC2 complex showed almost no immunoreactivity (both EED and EZH2 1% median expression; range 1-10 and range 1-5, respectively). Expression patterns of the PcG proteins were disturbed in ACC when compared to normal salivary gland tissue (Figure 2a-c). Members of the PRC1 complex were still abundantly expressed; BMI-1 exhibited a median expression of 95% (range 70-100) and median MEL-18 immunoreactivity increased to 70% (range 10-95). Interestingly, EED and EZH2 displayed significantly raised expression levels in the tumor (both $p<0.001$). Median expression of EZH2 increased to 10% (range 1-80) and in the case of EED to 35% (range 1-95).

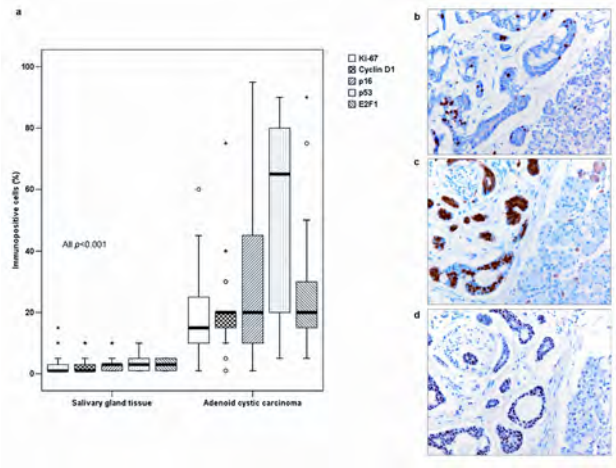

Figure 1 Significantly increased expression of the cell cycle proteins Ki-67, Cyclin D1, $p16^{INK4a}$, p53 and E2F1 in adenoid cystic carcinoma (ACC) compared to normal salivary gland tissue (all $p<0.001$). **(a)** Area of boxplot displays 50% of middle values; thick black line represents median number of immunopositive cells. Representative staining patterns of **(b)** Ki-67, **(c)** p16 and **(d)** p53 in normal salivary gland tissue (right side of picture) and in ACC (left side). Magnification, x200.

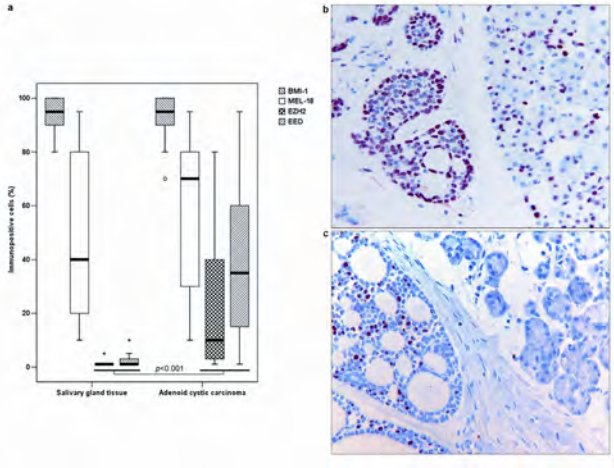

Figure 2 Significantly increased expression of members of the PRC2 complex in adenoid cystic carcinoma (ACC) compared to normal salivary gland tissue ($p<0.001$). **(a)** Area of boxplot displays 50% of middle values; thick black line represents median number of immunopositive cells. Representative staining patterns of **(b)** BMI-1 and **(c)** EZH2 in normal salivary gland tissue (right side of picture) and in ACC (left side). Magnification, x200.

Presence of locoregional recurrence and poor differentiation significantly correlate with poor patient outcome in ACC

To examine the influence of clinicopathological characteristics on patient outcome, we performed univariate survival analysis with sex, age (<55 vs ≥55 years), perineural spread, histological differentiation (solid vs cribriform/tubular), presence of recurrence, and presence of metastasis as prognostic factors. The presence of recurrence and the solid subtype significantly correlated with poor disease-specific survival rate (log rank=12.64, p=0.0004 and log rank=4.11, p=0.04, respectively; Table 2). No parameters showed significant association with disease-free survival.

Clinical parameter	n	Mean DSS (95% CI)	p Value	HR (95% CI)	X^2	p Value
Histologic subtype				0.2 (0.04-1.12)	3.63	0.05
Cribriform/tubular	14	115 (97-132)	0.04			
Solid	7	70 (49-91)	(LR=4.11)			
Recurrence				18.4	9.03	0.003
Absent	16	114 (97-132)	0.0004			
Present	5	58 (29-86)	(LR=12.64)			
EZH2				5.04 (0.91-27.82)	3.72	0.05
Low (≤ 25%)	15	115 (98-132)	0.04			
High (> 25%)	6	68 (45-91)	(LR=4.23)			

Table 2 Significant prognostic factors by univariate analysis. Abbreviations: DSS, disease-specific survival in months; CI, confidence interval; LR, log rank

Correlation of protein expression with clinical data

To correlate the expression of cell cycle and PcG proteins with categorical clinical data, we divided the expression rates into low (≤25%) and high (>25%) expression groups. All the proteins analyzed were correlated with sex and age of the patient, presence of recurrence or metastasis, patient outcome, histology (solid vs cribriform/tubular), and presence of perineural growth. Table 3 summarizes significantly correlating variables. Additionally, high $p16^{INK4a}$ and high Ki-67 expression showed a tendency towards more aggressive tumor behavior (p=0.08 and p=0.09, respectively), defined as development of either a recurrence or a metastasis. Correlation between individual proteins revealed that ACCs with high proliferative rate (>25% Ki-67 immunopositive cells) also displayed significantly higher EZH2 (p=0.05) and p16 levels (p=0.02). Additionally, tumors with low E2F1 status tended to express low amounts of EED (p=0.07). Univariate survival analysis classified EZH2 expression as a significant prognostic factor (log rank=4.23; p=0.04; Figure 3). However, multivariate survival analysis determined the presence of recurrence to be the only independent prognostic indicator (p=0.003).

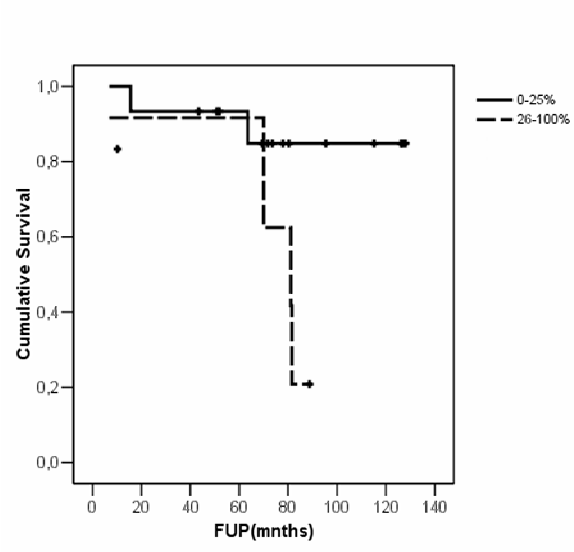

Figure 3 Overall survival curves of patients with adenoid cystic carcinoma showing low (≤25%; n=15) and high (>26%; n=6) EZH2 levels. A significant difference was observed between the two groups (log rank=4.23; p=0.04). FUP, follow-up

Clinical parameter	Total patients	Patients with low[a] Cyclin D1 staining (*n*=16)	Patients with high[b] Cyclin D1 staining (*n*=5)	*P* value
Metastasis				
Absent	10	5 (23.8%)	5 (23.8%)	0.01
Present	11	11 (52.4%)	0 (0%)	

Clinical Parameter	Total patients	Patients with low Ki-6a staining (*n*=16)	Patients with high[b] Ki-67 staining (*n*=5)	*P* value
Histology				
Cribriform/tubular	14	14 (66.7%)	0 (0%)	0.006
Solid	7	3 (14.3%)	4 (19%)	

Clinical Parameter	Total patients	Patients with low EZH2a staining (*n*=15)	Patients with high[b] EZH2 staining (*n*=6)	*P* value
Patient outcome				
Alive	15	13 (61.9%)	2 (9.5%)	0.03
Dead	6	2 (9.5%)	4 (19%)	

Table 3 Significant correlations between various cell cycle and Polycomb group (PcG) proteins and clinicopathologic parameters as determined by Fischer's exact test. [a] ≤25% of the cells immunopositive; [b] >25% of the cells immunopositive

DISCUSSION

Adenoid cystic carcinoma (ACC) is a malignant tumor of the salivary glands with an unpredictable clinical course. It has an unusually slow

biologic growth, resulting in a relatively favorable 5-year survival. About 30% of patients are alive after 15 years.[6] Since current prognostic indicators are not as reliable as desired, there is a need for novel molecular predictors of tumor behavior at the time of diagnosis. We assessed immunohistochemically the expression patterns of various cell cycle and Polycomb group (PcG) proteins in ACC and correlated our findings with patient follow-up data.

Univariate analysis of clinicopathologic characteristics in relation to patient survival revealed recurrence of the primary tumor and the solid histologic subtype as significant prognostic factors. Previous retrospective studies have also acknowledged the importance of histology concerning survival of patients with ACC.[25-27] The cribriform and tubular subtypes are considered to be more differentiated than the solid subtype; loss of differentiation is related to aggressive behavior in several cancer types.

Varambally *et al.* were the first to correlate increased *EZH2* expression with aggressive tumor behavior in hormone-refractory, metastatic prostate cancer and to show that clinically localized tumors with higher *EZH2* expression have a poorer prognosis.[28] Since then, raised EZH2 levels have been found in several epithelial tumours,[29-34] and in various haematological malignancies.[35-37] Studies which investigated the prognostic relevance of EZH2 revealed that high protein status predicted recurrence,[29] invasiveness and metastatic potential,[38] and poor survival.[29] In corroboration with this, high EZH2 (>25%) expression significantly correlated in our group of ACCs with poor patient outcome in univariate analysis, showing that in malignant tumors with myoepithelial differentiation PcG expression is disturbed and EZH2 is related to more aggressive tumor behavior. In multivariate analysis however, only recurrence remained as an independent prognostic factor.

It has been suggested that specific amplification of the *EZH2* gene at 7q35 is responsible for its overexpression in primary human tumors.[23] In our previous paper,[39] we investigated this same group of ACCs for frequent DNA aberrations by microarray-based comparative genomic hybridization. Although no amplifications were detected at 7q35, two tumors with high EZH2 protein expression displayed a chromosomal gain at its locus. Our findings imply that copy number elevations are not the main mechanisms of EZH2 overexpression; disturbances at other levels,

such as chromatin modification, or most likely a deregulation in the pRb-E2F pathway, are probably more important.

Recently, the finding that *EZH2* expression is critical for S-phase entry and G_2-M transition has implicated this PcG protein in cell cycle regulation.[23] *EZH2* transfection in lymphoma cells increased their proliferation,[37] and in cancers of the breast, endometrium and prostate and in cutaneous melanoma, EZH2 expression levels were strongly associated with increased tumor cell proliferation.[40] Similarly, Ki-67 expression in our group of ACCs showed a significant correlation with EZH2 expression ($p=0.05$).

After the discovery of the *INK4a* locus as a downstream target of Bmi-1 in murine models,[22] several *in vitro* studies have demonstrated the same negative regulation in human primary cells.[20, 41, 42] More specifically, EZH2 trimethylation of histone H3 on lysine 27 at the *INK4a* promoter and coding region serves as a mark to recruit the BMI-1-containing PRC1 complex. Binding of this complex to the locus prevents transcription by blocking the association of RNA polymerase II.[20] In our tumors, the observed high $p16^{INK4a}$ expression, even in the presence of BMI-1, suggests a deregulation of normal protein interactions. Two different explanations for this might be the impairment of BMI-1 function or the disruption of the pRb pathway downstream of p16. It has been discovered that in cases of overexpression/amplification of Cyclin D1 or mutation/deletion of pRb, p16 levels can accumulate as a result of oncogenic stress without causing proliferative arrest.[43, 44]

In conclusion, derailments in these essential cell cycle pathways on chromosomal and protein levels are very common in a wide range of human cancers,[44] and we speculate that similar alterations might be involved in ACC tumorigenesis.

Acknowledgments

The authors would like to thank Laura Jacobs for obtaining the patient data and W. de Jong for technical assistance. This work was supported financially by the Interuniversity Research School (IOT), Amsterdam, The Netherlands.

Chapter 3

REFERENCES

1. Spiro RH. Salivary neoplasms: overview of a 35-year experience with 2,807 patients. Head Neck Surg 1986;8:177-84.
2. Chaudhry AP, Leifer C, Cutler LS et al. Histogenesis of adenoid cystic carcinoma of the salivary glands. Light and electronmicroscopic study. Cancer 1986;58:72-82.
3. Caselitz J, Schulze I, Seifert G. Adenoid cystic carcinoma of the salivary glands: an immunohistochemical study. J Oral Pathol 1986;15:308-18.
4. Spiro RH, Huvos AG, Strong EW. Adenoid cystic carcinoma of salivary origin. A clinicopathologic study of 242 cases. Am J Surg 1974;128:512-20.
5. Therkildsen MH, Christensen M, Andersen LJ et al. Salivary gland carcinomas--prognostic factors. Acta Oncol 1998;37:701-13.
6. Kokemueller H, Eckardt A, Brachvogel P et al. Adenoid cystic carcinoma of the head and neck--a 20 years experience. Int J Oral Maxillofac Surg 2004;33:25-31.
7. Szanto PA, Luna MA, Tortoledo ME et al. Histologic grading of adenoid cystic carcinoma of the salivary glands. Cancer 1984;54:1062-9.
8. Alves FA, Pires FR, De Almeida OP et al. PCNA, Ki-67 and p53 expressions in submandibular salivary gland tumours. Int J Oral Maxillofac Surg 2004;33:593-7.
9. Cho KJ, Lee SS, Lee YS. Proliferating cell nuclear antigen and c-erbB-2 oncoprotein expression in adenoid cystic carcinomas of the salivary glands. Head Neck 1999;21:414-9.
10. Yamamoto Y, Wistuba II, Kishimoto Y et al. DNA analysis at p53 locus in adenoid cystic carcinoma: comparison of molecular study and p53 immunostaining. Pathol Int 1998;48:273-80.
11. Franchi A, Gallo O, Bocciolini C et al. Reduced E-cadherin expression correlates with unfavourable prognosis in adenoid cystic carcinoma of salivary glands of the oral cavity. Am J Clin Pathol 1999;111:43-50.
12. Freier K, Flechtenmacher C, Walch A et al. Differential KIT expression in histological subtypes of adenoid cystic carcinoma (ACC) of the salivary gland. Oral Oncol 2005;41:934-9.
13. Xue F, Zhang Y, Liu F et al. Expression of IgSF in salivary adenoid cystic carcinoma and its relationship with invasion and metastasis. J Oral Pathol Med 2005;34:295-7.
14. Zhu QR, White FH, Tipoe GL. p53 oncoprotein accumulation in adenoid cystic carcinoma of parotid and palatine salivary glands. Pathology 1997;29:154-8.
15. Yamamoto Y, Virmani AK, Wistuba II et al. Loss of heterozygosity and microsatellite alterations in p53 and RB genes in adenoid cystic carcinoma of the salivary glands. Hum Pathol 1996;27:1204-10.
16. Satijn DP, Otte AP. Polycomb group protein complexes: do different complexes regulate distinct target genes? Biochim Biophys Acta 1999;1447:1-16.
17. Lund AH, Van Lohuizen M. Polycomb complexes and silencing mechanisms. Curr Opin Cell Biol 2004;16:239-46.
18. Glinsky GV. Genomic models of metastatic cancer: functional analysis of death-from-cancer signature genes reveals aneuploid, anoikis-resistant, metastasis-enabling phenotype with altered cell cycle control and activated Polycomb Group (PcG) protein chromatin silencing pathway. Cell Cycle 2006;5:1208-16.
19. Raaphorst FM. Deregulated expression of Polycomb-group oncogenes in human malignant lymphomas and epithelial tumours. Hum Mol Genet 2005;14 Suppl 1:R93-R100.
20. Bracken AP, Kleine-Kohlbrecher D, Dietrich N et al. The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev 2007;21:525-30.
21. Jacobs JJ, Scheijen B, Voncken JW et al. Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. Genes Dev 1999;13:2678-90.

22. Jacobs JJ, Kieboom K, Marino S et al. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature 1999;397:164-8.
23. Bracken AP, Pasini D, Capra M et al. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J 2003;22:5323-35.
24. Oosterhuis JW, Coebergh JW, Van Veen EB. Tumour banks: well-guarded treasures in the interest of patients. Nat Rev Cancer 2003;3:73-7.
25. Huang M, Ma D, Sun K et al. Factors influencing survival rate in adenoid cystic carcinoma of the salivary glands. Int J Oral Maxillofac Surg 1997;26:435-9.
26. Fordice J, Kershaw C, El Naggar A et al. Adenoid cystic carcinoma of the head and neck: predictors of morbidity and mortality. Arch Otolaryngol Head Neck Surg 1999;125:149-52.
27. Matsuba HM, Spector GJ, Thawley SE et al. Adenoid cystic salivary gland carcinoma. A histopathologic review of treatment failure patterns. Cancer 1986;57:519-24.
28. Varambally S, Dhanasekaran SM, Zhou M et al. The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 2002;419:624-9.
29. Kleer CG, Cao Q, Varambally S et al. EZH2 is a marker of aggressive breast cancer and promotes neoplastic transformation of breast epithelial cells. Proc Natl Acad Sci U S A 2003;100:11606-11.
30. Raaphorst FM, Meijer CJ, Fieret E et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia 2003;5:481-8.
31. Breuer RH, Snijders PJ, Smit EF et al. Increased expression of the EZH2 polycomb group gene in BMI-1-positive neoplastic cells during bronchial carcinogenesis. Neoplasia 2004;6:736-43.
32. Sudo T, Utsunomiya T, Mimori K et al. Clinicopathological significance of EZH2 mRNA expression in patients with hepatocellular carcinoma. Br J Cancer 2005;92:1754-8.
33. Weikert S, Christoph F, Kollermann J et al. Expression levels of the EZH2 polycomb transcriptional repressor correlate with aggressiveness and invasive potential of bladder carcinomas. Int J Mol Med 2005;16:349-53.
34. Mimori K, Ogawa K, Okamoto M et al. Clinical significance of enhancer of zeste homolog 2 expression in colorectal cancer cases. Eur J Surg Oncol 2005;31:376-80.
35. Dukers DF, Van Galen JC, Giroth C et al. Unique polycomb gene expression pattern in Hodgkin's lymphoma and Hodgkin's lymphoma-derived cell lines. Am J Pathol 2004;164:873-81.
36. Van Kemenade FJ, Raaphorst FM, Blokzijl T et al. Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. Blood 2001;97:3896-901.
37. Visser HP, Gunster MJ, Kluin-Nelemans HC et al. The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br J Haematol 2001;112:950-8.
38. LaTulippe E, Satagopan J, Smith A et al. Comprehensive gene expression analysis of prostate cancer reveals distinct transcriptional programs associated with metastatic disease. Cancer Res 2002;62:4499-506.
39. Vékony H, Ylstra B, Wilting SM et al. DNA Copy Number Gains at Loci of Growth Factors and Their Receptors in Salivary Gland Adenoid Cystic Carcinoma. Clin Cancer Res 2007;13:3133-9.
40. Bachmann IM, Halvorsen OJ, Collett K et al. EZH2 expression is associated with high proliferation rate and aggressive tumour subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol 2006;24:268-73.
41. Guney I, Wu S, Sedivy JM. Reduced c-Myc signaling triggers telomere-independent senescence by regulating Bmi-1 and p16(INK4a). Proc Natl Acad Sci U S A 2006;103:3645-50.
42. Kotake Y, Cao R, Viatour P et al. pRB family proteins are required for H3K27 trimethylation and Polycomb repression complexes binding to and silencing p16INK4alpha tumour suppressor gene. Genes Dev 2007;21:49-54.

43. Kaye FJ. RB and cyclin dependent kinase pathways: defining a distinction between RB and p16 loss in lung cancer. Oncogene 2002;21:6908-14.
44. Michalides RJ. Cell cycle regulators: mechanisms and their role in aetiology, prognosis, and treatment of cancer. J Clin Pathol 1999;52:555-68.

CHAPTER 4

Copy Number Gain at 8q12.1-q22.1 is Associated with a Malignant Tumor Phenotype in Salivary Gland Myoepitheliomas

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Genes Chromosomes and Cancer, in press.

Chapter 4

ABSTRACT

Salivary gland myoepithelial tumors are relatively uncommon tumors with an unpredictable clinical course. More knowledge about their genetic profiles is necessary to identify novel predictors of disease. In this study, we subjected 27 primary tumors (15 myoepitheliomas and 12 myoepithelial carcinomas) to genome-wide microarray-based comparative genomic hybridization (array CGH). We set out to delineate known chromosomal aberrations in more detail and to unravel chromosomal differences between benign myoepitheliomas and myoepithelial carcinomas. Patterns of DNA copy number aberrations were analyzed by unsupervised hierarchical cluster analysis. Both benign and malignant tumors revealed a limited amount of chromosomal alterations (median of 5 and 7.5, respectively). In both tumor groups, high frequency gains (≥20%) were found mainly at loci of growth factors and growth factor receptors (e.g. *PDGF*, *FGF(R)s*, and *EGFR*). In myoepitheliomas, high frequency losses (≥20%) were detected at regions of proto-cadherins. Cluster analysis of the array CGH data identified three clusters. Differential copy numbers on chromosome arm 8q and chromosome 17 set the clusters apart. Cluster 1 contained a mixture of the two phenotypes (n=10), cluster 2 included mostly benign tumors (n=10), and cluster 3 only contained carcinomas (n=7). Supervised analysis between malignant and benign tumors revealed a 36 Mbp-region at 8q being more frequently gained in malignant tumors (p=0.007, FDR=0.05). This is the first study investigating genomic differences between benign and malignant myoepithelial tumors of the salivary glands at a genomic level. Both unsupervised and supervised analysis of the genomic profiles revealed chromosome arm 8q to be involved in the malignant phenotype of salivary gland myoepitheliomas.

INTRODUCTION

Myoepithelial cells are found in breast, salivary, sweat and lacrimal glands.[1] In salivary glands, they are normally located between the continuous luminal epithelial cells and the basal lamina of the acinar compartment.[1] Interestingly, these contractile cells exhibit a dual epithelial and myoid phenotype. Although they are an important element in many salivary gland tumors,[2] pure myoepithelial neoplasms are relatively rare. Benign myoepitheliomas (BMEs) comprise about 1.5% of all salivary gland tumors[3] and myoepithelial carcinomas (MMEs) are even less common.[4,5] On the basis of histopathologic examination alone, a definite diagnosis of benign or malignant myoepithelial tumor can be very difficult, e.g. when tumors are well circumscribed and encapsulated but have a high mitotic count, or remarkable cellular pleiomorphism. Cellular components in myoepitheliomas are polymorphous and include spindle-shaped, epithelioid, plasmacytoid, and clear cells or combinations thereof.[6] It has been proposed that these four cell types may represent different stages in myoepithelial cell differentiation.[7]

Defects in the maintenance of genome stability frequently result in DNA copy number alterations. Analysis of these genomic aberrations by microarray-based comparative genomic hybridization (array CGH) might provide a better understanding of the biologic behavior of myoepithelial tumors. However, there is limited amount of data on their underlying chromosomal changes. Three contributing factors to this might be that, (1) myoepithelial tumors are relatively uncommon, (2) they lack defined precursor lesions, and (3) they are difficult to culture for cytogenetic analysis and cell line development. However, the few cytogenetic reports on these tumors have provided important background information about their frequently aberrated chromosomal regions.[8-13] Compared to most other solid epithelial tumors, myoepithelial neoplasms in salivary and breast tissue seem to have fewer chromosomal aberrations. One conventional cytogenetic study reported a normal karyotype in a benign myoepithelioma[8] and El-Naggar *et al.* and Hungermann *et al.* found only a few chromosomal aberrations.[10,12] Surprisingly, myoepithelial carcinomas in salivary and breast tissue also revealed a paucity of genomic alterations.[11,12] Gains were more frequent than losses and amplifications, if present, were detected at the 12q region. Most

prevalent DNA copy number increases were of chromosome arms 8q (26%) and 1q (21%), and of chromosome 5 (21%). The scarceness of genomic alterations in these lesions might be explained by the relative quiescent status of myoepithelial cells or their suggested greater ability to repair DNA.[14]

In this study, we analyzed the chromosomal profiles of a relatively large group of myoepithelial tumors of the salivary glands (15 benign and 12 malignant) by array CGH. This technique allowed a high resolution, genome-wide overview of tumor DNA isolated from archival material.[15] In addition to identifying commonly altered regions, we correlated our findings with malignant phenotype to define chromosomal areas discriminating benign from myoepithelial carcinomas.

MATERIALS AND METHODS

Patient Samples

The formalin-fixed, paraffin-embedded (FFPE) tissue samples of myoepithelial tumors, diagnosed from 1991 to 2007, were derived from two sources: 15 myoepitheliomas and 4 myoepithelial carcinomas were retrieved from the archives of the Department of Pathology at the VU University Medical Center in Amsterdam, the Netherlands. Histological diagnosis thereof was reviewed independently by two pathologists (EB and IvdW). Additionally, 8 myoepithelial carcinomas were obtained from the files of the Salivary Gland Registry at the Department of Oral Pathology, University of Hamburg, Germany. Another experienced salivary gland pathologist (TL) confirmed the diagnoses of these tumors. Criteria of malignancy consisted of invasive growth or necrosis with considerable polymorphism of the cells with high mitotic index. After haematoxylin and eosin (H&E) staining, all but one tumor specimens were evaluated and subtyped according to cell type. The majority of BMEs was of spindle cell histology (n=10), four benign tumors were epithelioid, and one case was plasmacytoid. In the MMEs, four tumors were plasmacytoid, four were spindle cell, one was a mixture of these two types, one had clear cell features, and one was epithelioid.

For all Dutch cases (*n*=19), patient follow-up data were available. In case of the German tumors, only the gender of the patient could be retrieved. The male to female ratio was 1:1.5 for the BMEs and 1:1.2 for the MMEs. Average age at diagnosis for the BMEs was 59 years (range, 39-75). Mean age at diagnosis for the Dutch malignant cases (*n*=4) was 47 years (range, 32-60). Thirteen BMEs were located in the major salivary glands (parotid *n*=11, submandibular *n*=2), and two benign tumors were located in the parapharyngeal space. Four MMEs were located in the parotid gland, one was found in a minor salivary gland and in seven cases information was missing. None of the Dutch cases (*n*=19) developed a recurrence or a metastasis during the time of follow-up (FUP). For the BMEs, mean FUP time was 44 months (range, 0-134) and an average of 59 months (range, 8-111) was the FUP for the MMEs. All tumors were surgically resected, and two malignant tumors received post-operative radiation. One patient died during FUP, not due to the tumor.

DNA extraction

Of each paraffin block, haematoxylin and eosin (H&E) stainings were reviewed to assure that at least 70% tumor cells were present before sectioning and DNA extraction. Genomic DNA was extracted from three 10 µm thick FFPE tissue sections per case. After deparaffinating with xylene and rehydrating with decreasing concentrations of ethanol (100% - 96% - 70%), the sections were haematoxylin stained and tumor areas were macrodissected with a surgical blade. DNA extraction was performed according to standard protocols of proteinase K (1mg/ml) digestion overnight, followed by phenol-chloroform extraction.

Microarray-based comparative genomic hybridization (array CGH)

6k BAC arrays

CGH BAC microarrays were produced either at the Microarray facility of the VU University Medical Center using an OmniGrid® 100 microarrayer (Genomic Solutions, Ann Arbor, MI, USA) or at the University Medical Centre Groningen (UMCG) using a MicroGrid II arrayer (BioRobotics, Cambridge, UK). The VUmc arrays included a total of 5755 BAC clones

(average spatial resolution of 1 Mb)[16] and the Groningen arrays consisted of 6465 BAC clones, both with known chromosomal locations (average spatial resolution of 1 Mb).[17] After extraction, DNA quality was assessed by isothermal amplification, as described previously.[18] Only DNAs which scored as excellent, good, or intermediate were used for array CGH analysis. Genomic DNA (600 ng) from both the test and reference sample was labeled and hybridizations were essentially performed according to Snijders *et al.*[19] without the prehybridization step. As reference DNA, normal DNA pooled from several individuals from the same sex was employed, since our normal matched FFPE material was not sufficient to be used as standard reference material. The epoxy-coated slides (Schott Nexterion, Mainz, Germany) used for the Groningen arrays were processed as recommended by the manufacturer. Hybridization solution was added to the array in a hybridization station (HybArray12™, Perkin Elmer Life Sciences, Zaventum, Belgium) and incubated for 38 h at 37°C.

44k oligo arrays

To explore the 12q amplicon in more detail, a 44k oligonucleotide array was employed. This platform included 44,000 60-mer oligonucleotides allowing a genome-wide survey and molecular karyotyping of genomic aberrations with an average resolution of 75 kb.[20] Labeling of tumor and reference DNA was performed using the ENZO BioArray™ CGH Labeling System (Farmingdale, NY, USA). 500 ng genomic DNA of tumor was labeled with Cy3 and mixed with a normal human reference pool of ten healthy individuals (labeled with Cy5) prior to hybridization on a 4x44K Agilent slide (Amstelveen, The Netherlands) using the Agilent hybridization oven (G2545A) overnight at 20 rpm. Washing, scanning and feature selection (Feature extraction software v.9.1) were furthermore performed using standard Agilent procedures. Data presented include all spots printed on the arrays and are available via GEO series accession number GSE12951.

Image and data processing

BAC and oligo CGH-microarray slides were scanned using a DNA microarray scanner G2565AB (Agilent Technologies Netherlands B.V.).

Spot analysis and quality control for the BAC arrays were fully automated using Bluefuse 3.4 edition software (BlueGnome, Cambridge, UK). Spots with Quality flag <1 or Confidence value <0.3 were excluded. Log_2ratios (tumor signal divided by normal reference signal) of spots that were not excluded after quality flagging and mapping were normalized to their block median value.

For the array analysis, BAC clones were positioned along the genome using the March 2006 freeze of the UCSC database. For each clone, the average of the triplicate spots was calculated and ratios were normalized by subtraction of the median value of all BAC clones on chromosomes 1-22. In order to determine exact breakpoints in the generated array CGH profiles, we segmented the obtained log_2 ratios by DNAcopy,[21] which was shown to be one of the strongest segmentation algorithms.[22] The sex chromosomes were discarded from the analysis, since all tumor samples were hybridized to reference DNA of the opposite gender.

Statistical analysis

In the downstream analysis, only clones with less then 30% missing values were included. Segmented log_2 ratios were converted to four levels of categorized data (i.e. losses, normals, gains, and amplifications) by CGHcall.[23] To reduce the dimension of the data set, regions of DNA copy number changes were constructed by an algorithm called CGHregions,[24] implemented using the statistical software environment R [R Development Core Team (2006)]. In our settings, we accepted maximally 1% information loss (T=0.01). Array CGH data were analyzed both in an unsupervised and in a supervised manner. For unsupervised data analysis, regions of DNA copy number changes of all cases were analyzed by hierarchical cluster analysis using WECCA (weighted clustering of called aCGH data).[25] WECCA allows the user to assign weights to each region, determining their relative influence on the clustering. To reflect differences in the regions' coverage of the genome, we chose a region's weight equal to the number of clones it contains. WECCA then builds a dendrogram with the concordance similarity and total linkage. The latter results in compact, well-separated clusters. Finally, to determine the regions that are most distinct between clusters, we calculated (per region) the maximum pair wise symmetrized

Kullback Leibler divergence.[26] Regions with the highest divergence (i.e. distance) differ most in their copy number distribution between clusters. For supervised analysis, differential chromosomal loci between benign myoepitheliomas and myoepithelial carcinomas were identified by a Wilcoxon two-sample test corrected for ties (i.e. equal copy numbers were considered as ties).[27] With this statistical test, the ordinality of the four copy number states in the tumors (i.e. losses, normals, gains, and amplifications) is taken into account, which was proven to be the most powerful method. Robustness of the findings was also assured by including a false discovery rate (FDR) correction for multiple testing, needed to discriminate real differences from chance effects. Two-sided FDR values of ≤0.05 were considered statistically significant; meaning that maximally one out of 20 claimed discoveries was expected to be false. Associations between cluster assignment and tumor malignancy were performed by the Chi square statistical test.

RESULTS

Limited amount of chromosomal aberrations in benign myoepitheliomas

Aberrant chromosomal areas were detected in all benign myoepitheliomas (n=15), with a total of 98 genomic alterations (Figure 1a). Median amount of copy number changes (gains, losses and amplifications) was 5 (range, 0-30). Gains were more common than losses in these benign tumors (63 and 31, resp.). Furthermore, one amplification at locus 6q23.3 was detected in four different tumors (Table 1). The most recurrent copy number gains were of 22q11.1-q13.33 and 11q23.3 in 40% and 38% of the tumors respectively. The frequent losses occurred at relatively small regions, namely at 15q11.2 in 33% and at 20p11.1-q11.21 in 40% of the myoepitheliomas. Candidate oncogenes and tumor suppressor genes in these frequently altered regions were identified by consulting the Entrez Gene database at the National Center for Biotechnology.

	Cytoband	Size of region (Mbps)	Percentage of tumors (%)	Candidate genes
Gains	7p11.2	0.8	27	*EGFR*
	11q23.3	2.5	38	*MLL*, *CBL*, *DDX6*, *BCL9L*, *MCAM*, *RNF26*, *HYOU1*
	19p13.3-q13.43	63.6	20	*FGF21*, *FGF22*, *DDX39*
	22q11.1-q13.33	33.8	40	*BCR*, *CRKL*, *PDGFB*
Losses	10p11.21-q11.22	10.5	20	*RASSF4*
	13q21.1-q21.33	17.1	20	*PCDH9*, *PCDH17*, *PCDH20*
	15q11.2	0.8	33	*POTE15*
	20p11.1-q11.21	3.6	40	*MGC72104*
Ampl	6q23.3	0.18	27	*PDE7B*

Table 1 Most frequently (≥ 20%) gained and lost minimal common regions of overlap and amplifications detected by microarray based comparative genomic hybridization in 15 primary myoepitheliomas; together with candidate oncogenes and tumor suppressor genes. Abbreviations: Ampl – amplification; Mbps - Megabasepares

Interestingly, high level gains (in ≥20% of the tumors) were observed at the loci of growth factors or growth factor receptors [i.e. fibroblastic growth factors (*FGFs*) and platelet-derived growth factor beta (*PDGFB*), and epidermal growth factor receptor (*EGFR*)], and several regulatory factors (e.g. *MLL*, *DDX6*), which are known to be important cancer-related genes. Interestingly, recurrent losses occurred at the chromosomal sites of proto-cadherins (i.e. *PCDH9*, *17* and *20* at chromosome 13 in 20% of the tumors). The single amplification in our cohort of myoepitheliomas, although detected in four different tumors, contained only the phosphodiesterase 7B (*PDE7B*) gene.

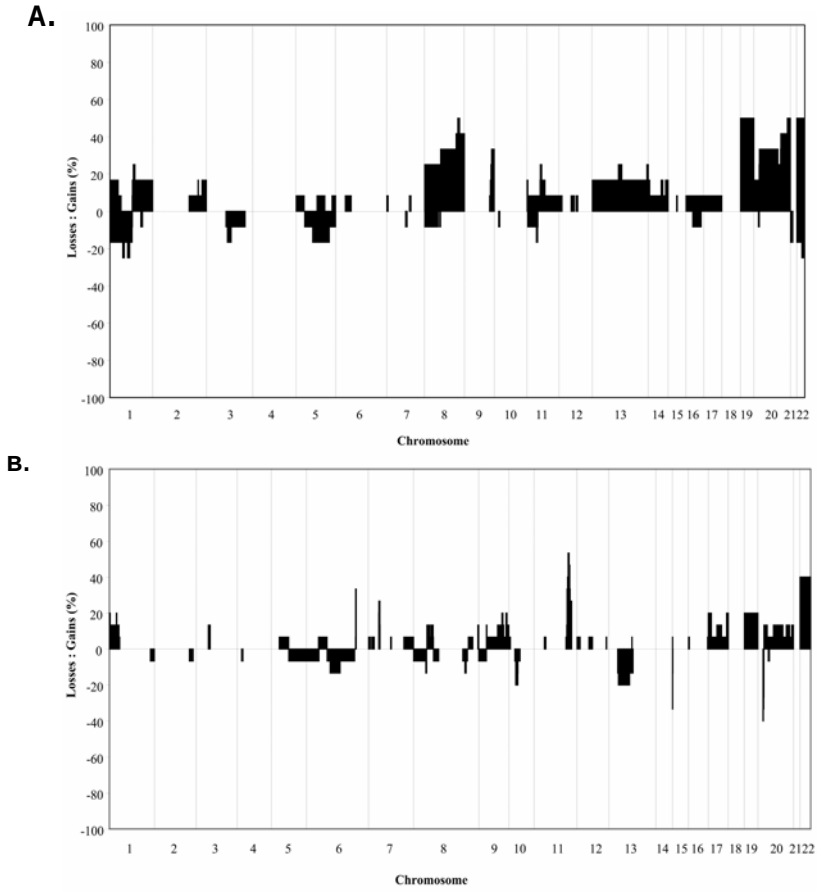

Figure 1 Array CGH copy number frequency plots for chromosomes 1-22. Percentages of gains and amplifications (positive axis) and losses (negative axis) are shown for each BAC clone for **(A)** all myoepitheliomas and for **(B)** all myoepithelial carcinomas.

Myoepithelial carcinomas show frequent gains of whole chromosomes and chromosomal arms

In general, myoepithelial carcinomas (*n*=12) displayed slightly more chromosomal events than their benign counterparts (a total of 86, median of 7.5, range, 1-18; Figure 1b). Again, gains were more prevalent than losses (60 vs. 21, resp.). High-level amplifications were uncommon (*n*=5) and did not include the 6q23.3 amplicon found in the benign counterparts. Most frequently altered chromosomal areas are summarized in Table 2. The most common gains include 19p13.3-q13.43 and 22q11.1-q13.33 (both 50%), 20q11.21-q13.33 (32%), 9q33.3-q34.3 (29%), and 8p23.3-q24.3 (27%). Merely two recurrent losses were detected in this group of tumors, namely loss of 1p31.1 and 1p21.3-p21.2 (both 25%). As in the benign tumors, high level gains (≥20%) occurred at the loci of growth factors, like *PDGFB* and numerous FGFs (*FGF3*, *4*, *19*, *17*, *20*, *21*, *22*, and *FGFR1*). A high frequency of copy number increases involving whole chromosomes (19 and 8) or chromosomal arms (20 and 22) was observed. In two tumors, the 12q14.3-q21.1 area was amplified. Since the 12q14-15 region has been reported to be frequently aberrated in various human tumors,[28-30] we hybridized one of our two myoepithelial carcinomas containing this amplification to a high-resolution 44k oligo array to investigate the aberration in more detail. The spotted $\log_2$ratios along chromosome 12 are shown in Figure 2a and a detailed view of the amplified areas is depicted in Figure 2b. Three amplicons were detected in this region, namely (#1; 0.1 Mbps) from 56373676 to 56516893 harboring 10 genes, amongst which the cyclin dependent kinase 4 gene (*CDK4*) and the sarcoma amplified sequence (*TSPAN31*), (#2; 0.7 Mbps) from 63988612 to 64727853 encompassing two genes, one of the two being the high-mobility group AT-hook 2 (*HMGA2*) gene, and (#3; 1.6 Mbps) from 67369576 to 68958280 harboring ten genes, the most interesting being the mouse double minute 2 (*MDM2*) gene. The complete list of the genes located in the three amplicons is summarized in Table 3.

	Cytoband	Size of region (Mbps)	Percentage of tumors (%)	Candidate genes
Gains	1q21.2-q22	5.9	25	*S100, MUC1, ADAM15*
	8p23.3-q24.3	145.8	27	*FGF17, FGF20, FGFR1, MMP16*
	9q33.3-q34.3	13.4	29	*ABL1, DDX31, VAV2*
	11q12.2-q13.5	15.3	25	*RELA,FGF3,FGF4,FGF19*
	13q21.33-q22.1	3.4	25	*KLF5*
	13q34	4.9	25	*TFDP1, SOX1, RAB20*
	19p13.3-q13.43	63.6	50	*FGF21, FGF22, DDX39*
	20q11.21-q13.33	33.1	32	*PTPN1, PLAGL2, MMP24, BCAS1*
	22q11.1-q13.33	33.7	50	*BCR, CRKL, PDGFB*
Losses	1p31.1	9.3	25	*NEGR1*
	1p21.3-p21.1	11.1	25	*EDG1*
Amplifications	1p32.2-p31.3	3.9	8	*JUN, TACSDT2*
	5p15.33-p13.2	35.1	8	*TERT*
	12q14.3-q21.1	7.4	17	*HMGA2, MDM2, RAB21, PTPRB*
	20q13.2	0.5	8	*DOK5, PFDN4*

Table 2 Most frequently (≥ 20%) gained and lost minimal common regions of overlap and amplifications detected by microarray based comparative genomic hybridization in 12 primary myoepithelial carcinomas; together with candidate oncogenes and tumor suppressor genes. Abbreviations: Mbps, mega-basepares

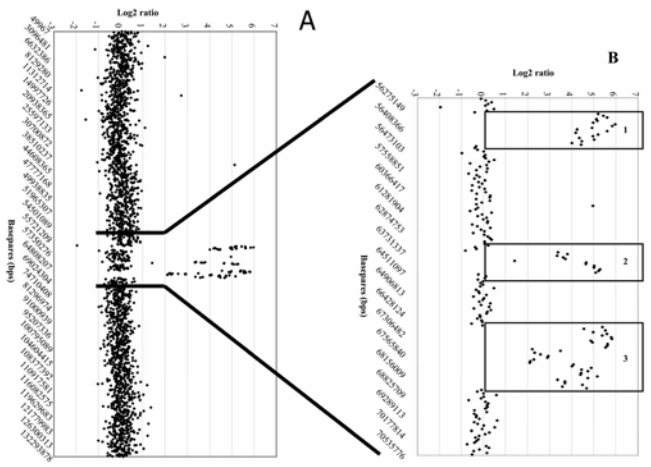

Figure 2 (A) Array CGH plot of chromosome 12 in a myoepithelial carcinoma showing the amplification at the 12q14.3-q21.1 region. **(B)** Detailed view of the three amplicons at chromosome 12.

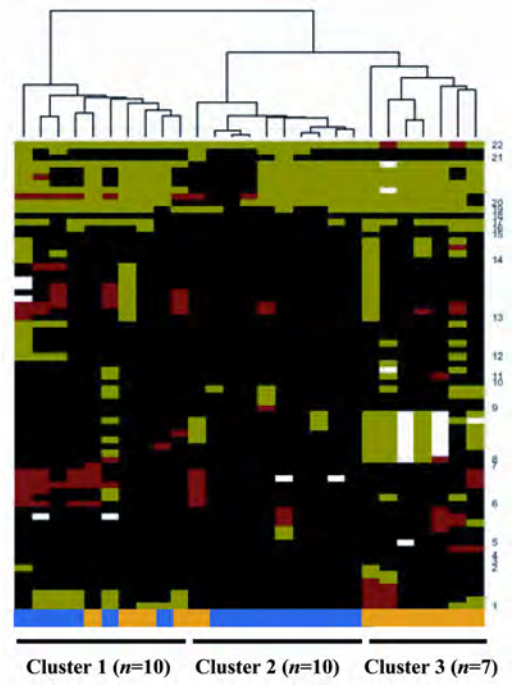

Figure 3 Hierarchical cluster analysis of the array CGH data of 27 benign and malignant myoepitheliomas revealed three clusters. Cluster 1 was a heterogeneous group, containing 4/12 carcinomas and 6/15 benign tumors, cluster 2 was mainly benign, containing 9/15 myoepitheliomas and 1 carcinoma, and cluster 3 contained seven tumors, all of which were malignant. The columns represent the different tumors and the rows represent the different genomic regions after analysis with CGHregions. Yellow and blue boxes below the dendrogram represent malignant and benign tumors, respectively.

Amplicon 1 56373676-56516893	Amplicon 2 63988612-64727853	Amplicon 3 67369576-68958280
OS9	*HMGA2*	*NUP107*
TSPAN31	*MSRB3*	*CPM*
TSFM		*CPSF6*
CTDSP2		*FRS2*
AVIL		*RAB31P*
CDK4		*CCT2*
CENTG1		*BEST3*
METTL1		*LYZ*
CYP27B1		*MDM2*
FAM119B		*YEATS4*

Table 3 Complete list of the genes located in the three amplicons detected at the chromosomal region 12q14.3-q21.1 in a myoepithelial carcinoma.

Differential genomic alterations in benign myoepitheliomas and myoepithelial carcinomas

Previous studies have observed that patterns of gains and losses harbor a level of tumor specificity.[31] We wanted to explore whether new subclasses of our myoepithelial tumors would be formed based on patterns of their chromosomal aberrations. Consequently, we performed hierarchical cluster analysis of the array CGH regions of our 27 tumors by the WECCA method. Regions were weighted according to their size (i.e. larger regions were assigned a weight proportional to the number of array elements they covered). Three clusters with different genomic profiles were detected (Figure 3). Cluster 1 was a mixture of malignant (4/12) and benign (6/15) tumors, cluster 2 contained mostly benign (9/15) myoepitheliomas and only 1 malignant tumor, and cluster 3 consisted only of myoepithelial carcinomas. Regions with most differential copy numbers between cluster 2 (“benign”) and cluster 3 (“malignant”) comprised five regions on chromosome 8 (i.e. 8q12.1-q22.1, 8q22.1-q22.3, 8q22.3-q23.3, 8q23.3-q24.13, and 8q24.21-q24.3) and the entire chromosome 17 (Figure 4). Association between cluster assignment and tumor malignancy was highly significant (p=0.001).

Myoepithelial carcinomas are characterized by a significantly differential copy number increase of region 8q12.1-q22.1

By supervised analysis, dividing our tumors in a benign and a malignant group, the most significantly differential area (FDR=0.05) comprised a ~36 Mbps-region on chromosome 8. This chromosomal area (8q12.1-q22.1) contained 99 known genes and was gained in 50% (6/12) of the myoepithelial carcinomas while in the benign tumors this gain was absent (0/15).

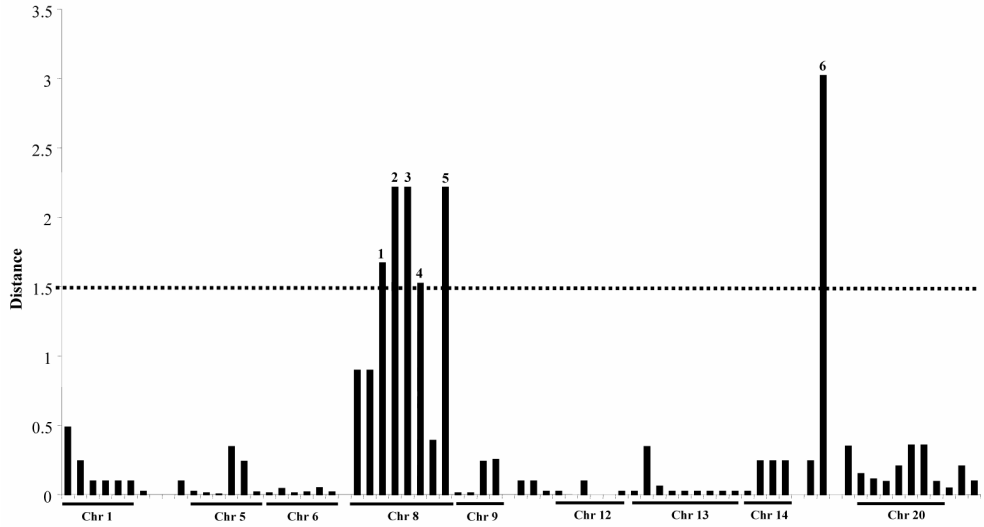

Figure 4 Divergence (i.e. distance) of chromosomal regions between clusters is plotted. Distances with a value above 1.5 were considered as highly discriminating. Five almost adjacent regions at chromosome 8 and the entire chromosome 17 meet this criterion. Numbers on top of bars represent these significantly differential regions, namely (1) - 8q12.1-q22.1; (2) - 8q22.1-q22.3; (3) - 8q22.3-q23.3; (4) - 8q23.3-q24.13; (5) - 8q24.21-q24.3 and (6) - 17p13.3-q25.3.

Chapter 4

DISCUSSION

Although tumors with a myoepithelial cell component have been recognized for some time,[32,33] studies focusing on their chromosomal profiles are scarce. To extend our knowledge on the genomic background of myoepithelial tumors of the salivary glands, we performed a genome-wide analysis of their DNA copy number alterations by array CGH.

In concordance with earlier studies,[10,12] we have found relatively few aberrations in both benign myoepitheliomas and myoepithelial carcinomas (median of 5 for benign, and median of 7.5 for malignant tumors). This finding is probably more tumor than tissue specific, since myoepithelial carcinomas of the breast also displayed a low number of alterations compared to other solid tumors.[11] As in our previous study on salivary gland adenoid cystic carcinomas (ACCs), gains were more common in both benign myoepitheliomas and myoepithelial carcinomas than losses, and the amount of amplifications was limited.[16]

The most frequent gains in the benign tumors were detected at 22q11.1-q13.33 (40%) and 11q23.3 (38%). The latter is a very gene-rich region, and is involved in recurrent genomic aberrations in various cancers.[16,34,35] Genes of interest in this locus are the oncogenes *MLL*, *CBL*,[34] and *BCL9L*.[36] In ~30% of the tumors a specific copy number increase of the *EGFR* gene (at 7p11.2) was observed. Moreover, loci of other growth factors (FGF21 and 22, and PDGFB) were also detected at regions of recurrent gains. Involvement of FGFs and PDGF in salivary gland tumor development and progression has been implicated previously by us,[16] and others.[37,38] Recurrent losses (20%) were observed at the loci of several proto-cadherins (PCDH9, 17 and 20 at 13q21.1-q21.33). Proto-cadherins are a subfamily of cadherins[39] which mediate adhesion in synaptic junctions. Mutated, deleted or methylated versions of several cadherin family members have been associated with loss of differentiation, acquisition of an invasive phenotype, and an unfavorable prognosis in various types of cancer.[40-42] We detected merely one amplification in our group of myoepitheliomas. Interestingly, this specific amplification of the 6q23.3 region was not only seen in four different tumors, but the area also contained only a single gene, namely phosphodiesterase 7B (PDE7B). PDE7 has been shown to be essential

for induction of T-cell proliferation,43 and its deregulated expression has been correlated with allergies and chronic inflammation.[44]

In general, carcinomas displayed more chromosomal events than benign tumors. One striking difference between the genomic profiles of myoepithelial carcinomas and their benign counterparts were the recurrent gains encompassing large genomic regions. High frequency copy number gains of whole chromosomes (chr. 8 in 27% and chr. 19 in 50%) or chromosomal arms (20q in 32% and 22q in 50%) were detected. Gains corresponding to the entire chromosome 8 have not only been observed in myoepithelial tumors[12] of the salivary glands, but also in salivary adenocarcinomas and mucoepidermoid carcinomas.[45,46] Furthermore, rearrangements and amplifications on this chromosome are a recurrent event in pleiomorphic adenomas (PAs), in particular involving translocations of the *PLAG1* gene.[47] Region 9q33.3-q34.3, harboring several oncogenes (e.g. *ABL1* and *VAV2*), was gained in about one-third of the carcinomas. Furthermore, loci of several FGFs, including FGFR1, were abundantly gained in our group of myoepithelial carcinomas. Losses do not seem to play a major role in myoepithelial tumorigenesis. In the malignant neoplasms, we observed a median loss of 1 and only two deletions occurred at a high frequency (25%). The neuronal growth regulator 1 (*NEGR1* at 1p31.1) and estrogen down-regulated gene 1 (*EDG1* at 1p21.3-p21.1) are candidate tumor suppressor genes at these 1p regions. *NEGR1* expression is reduced in ovarian cancer, thus it might function as a tumor suppressor.[48] *EDG1* is novel inhibitor of breast cell proliferation and shows downregulated expression in human breast cancer tissue.[49]

Amplifications in our carcinomas, although uncommon (n=5), were found at interesting chromosomal regions, like 12q and 20q. Rearrangements of 12q are a common finding in human mesenchymal tumors,[50] as well as in salivary gland pleiomorphic adenomas[51] and myoepithelial carcinomas. The target gene in this region is usually *HMGA2*, a DNA-binding factor involved in many fundamental processes, including gene regulation, cell cycle and differentiation.[52] Using high-resolution oligonucleotide array CGH, we were able to characterize the 12q amplification in more detail. Importantly, amplification of this genomic region, more specifically of the genes *CDK4*, *MDM2* and *HMGA2*, has been detected in carcinoma ex PA and correlated with the acquisition of a malignant phenotype.[53,54] In concordance with these

studies, we detected three amplicons, including *CDK4* and *MDM2* and a specific amplification of *HMGA2*. Copy number gains and amplifications of the 20q13.2 region have been observed in several types of cancer, especially in breast tumors.[55] Our 20q-amplicon contained two genes: *DOK5*, an adaptor protein involved in signal transduction and *PFDN4*, a member of the prefoldin beta subunit family.

Unsupervised cluster analysis revealed three clusters with a clear correlation to a benign or malignant phenotype. Clusters differed mostly on five regions on chromosome 8q and on the entire chromosome 17. All these chromosomal segments displayed a higher frequency of gains in the myoepithelial carcinomas. In addition to the unsupervised clustering, supervised analysis also detected regions on chromosome 8q to differ significantly between malignant and benign tumors, indicating even more, that genes in this region might contribute to malignant transformation. Interesting candidate genes are *RAB2A*, a member of the RAS oncogene family, the matrix metalloproteinase *MMP16*, the tumor protein *TPD52*, the pituitary tumor transforming gene 3 (*PTTG3*), and the myelogenous leukemia 1 translocation 1 protein (*RUNX1T1*).

Acknowledgments

We thank the mapping core and map finishing groups of the Wellcome Trust Sanger Institute for initial BAC clone supply and verification. We are indebted to A. Mrsic, P.P. Eijk, T. Krugers, and W. de Jong for technical assistance. We acknowledge S. Wilting for her help with the data analysis.

REFERENCES

1. Hamperl H. 1970. The myothelia (myoepithelial cells). Normal state; regressive changes; hyperplasia; tumors. Curr Top Pathol 53:161-220.
2. Dardick I. 1985. A role for electron microscopy in salivary gland neoplasms. Ultrastruct Pathol 9:151-161.
3. Barnes L, Eveson JW, Reichart P, Sidransky D. 2005. World Health Organization Classification of Tumours: Pathology and Genetics of Head and Neck Tumours. Lyon: IARCPress. p 259-260.
4. Sciubba JJ, Brannon RB. 1982. Myoepithelioma of salivary glands: report of 23 cases. Cancer 49:562-572.
5. Ellis GL, Auclair PL. 1996. Tumors of the Salivary Glands. Atlas of tumor pathology, 3rd series, fasicle 17. Washington D.C.: Armed Forces Institute of Pathology. 266 p.
6. Dardick I, Cavell S, Boivin M, Hoppe D, Parks WR, Stinson J, Yamada S, Burns BF. 1989. Salivary gland myoepithelioma variants. Histological, ultrastructural, and immunocytological features. Virchows Arch A Pathol Anat Histopathol 416:25-42.
7. Da Silveira EJ, Pereira AL, Fontora MC, De Souza LB, De Almeida FR. 2006. Myoepithelioma of minor salivary gland--an immunohistochemical analysis of four cases. Rev Bras Otorrinolaringol (Engl Ed) 72:528-532.
8. Martins C, Fonseca I, Felix A, Roque L, Soares J. 1995. Benign salivary gland tumors: a cytogenetic study of 21 cases. J Surg Oncol 60:232-237.
9. Martins C, Fonseca I, Roque L, Pinto AE, Soares J. 1996. Malignant salivary gland neoplasms: a cytogenetic study of 19 cases. Eur J Cancer B Oral Oncol 32B:128-132.
10. El Naggar AK, Lovell M, Callender DL, Ordonez NG, Killary AM. 1999. Cytogenetic analysis of a primary salivary gland myoepithelioma. Cancer Genet Cytogenet 113:49-53.
11. Jones C, Foschini MP, Chaggar R, Lu YJ, Wells D, Shipley JM, Eusebi V, Lakhani SR. 2000. Comparative genomic hybridization analysis of myoepithelial carcinoma of the breast. Lab Invest 80:831-836.
12. Hungermann D, Roeser K, Buerger H, Jakel T, Loening T, Herbst H. 2002. Relative paucity of gross genetic alterations in myoepitheliomas and myoepithelial carcinomas of salivary glands. J Pathol 198:487-494.
13. Magrini E, Pragliola A, Farnedi A, Betts CM, Cocchi R, Foschini MP. 2004. Cytogenetic analysis of myoepithelial cell carcinoma of salivary gland. Virchows Arch 444:82-86.
14. Lakhani SR, O'Hare MJ. 2001. The mammary myoepithelial cell--Cinderella or ugly sister? Breast Cancer Res 3:1-4.
15. De Vries S, Nyante S, Korkola J, Segraves R, Nakao K, Moore D, Bae H, Wilhelm M, Hwang S, Waldman F. 2005. Array-based comparative genomic hybridization from formalin-fixed, paraffin-embedded breast tumors. J Mol Diagn 7:65-71.
16. Vékony H, Ylstra B, Wilting SM, Meijer GA, Van de Wiel MA, Leemans CR, Van der Waal I, Bloemena E. 2007. DNA Copy Number Gains at Loci of Growth Factors and Their Receptors in Salivary Gland Adenoid Cystic Carcinoma. Clin Cancer Res 13:3133-3139.
17. Atayar C, Kok K, Kluiver J, Bosga A, Van den Berg E, Van der Vlies P, Blokzijl T, Harms G, Davelaar I, Sikkema-Raddatz B, Martin-Subero JI, Siebert R, Poppema S, Van den Berg A. 2006. BCL6 alternative breakpoint region break and homozygous deletion of 17q24 in the nodular lymphocyte predominance type of Hodgkin's lymphoma-derived cell line DEV. Hum Pathol 37:675-683.
18. Buffart TE, Tijssen M, Krugers T, Carvalho B, Smeets SJ, Brakenhoff RH, Grabsch H, Meijer GA, Sadowski HB, Ylstra B. 2007b. DNA quality assessment for array CGH by isothermal whole genome amplification. Cell Oncol 29:351-359.
19. Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Huey B, Kimura K, Law S, Myambo K, Palmer J, Ylstra B, Yue JP, Gray JW,

Jain AN, Pinkel D, Albertson DG. 2001. Assembly of microarrays for genome-wide measurement of DNA copy number. Nat Genet 29:263-264.

20. Coe BP, Ylstra B, Carvalho B, Meijer GA, MacAulay C, Lam WL. 2007. Resolving the resolution of array CGH. Genomics 89:647-653.
21. Olshen AB, Venkatraman ES, Lucito R, Wigler M. 2004. Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics 5:557-572.
22. Willenbrock H, Fridlyand J. 2005. A comparison study: applying segmentation to array CGH data for downstream analyses. Bioinformatics 21:4084-4091.
23. Van de Wiel MA, Kim KI, Vosse SJ, Van Wieringen WN, Wilting SM, Ylstra B. 2007. CGHcall: calling aberrations for array CGH tumor profiles. Bioinformatics 23:892-894.
24. Van de Wiel MA, Van Wieringen WN. 2007. CGHregions: Dimension reduction for Array CGH data with minimal information loss. Cancer Informatics 2:55-63.
25. Van Wieringen WN, Van de Wiel MA, Ylstra B. 2008. Weighted clustering of called array CGH data. Biostatistics 9: 484-500.
26. Tumminello M, Lillo F, Mantegna RN. 2007. Kullback-Leibler distance as a measure of the information filtered from multivariate data. Phys Rev E Stat Nonlin Soft Matter Phys 76:031123.
27. Van de Wiel MA, Smeets SJ, Brakenhoff RH, Ylstra B. 2005. CGHMultiArray: exact p-values for multi-array CGH-data. Bioinformatics 21: 3193-3194.
28. Bullerdiek J, Wobst G, Meyer-Bolte K, Chilla R, Haubrich J, Thode B, Bartnitzke S. 1993. Cytogenetic subtyping of 220 salivary gland pleomorphic adenomas: correlation to occurrence, histological subtype, and in vitro cellular behavior. Cancer Genet Cytogenet 65:27-31.
29. Odero MD, Grand FH, Iqbal S, Ross F, Roman JP, Vizmanos JL, Andrieux J, Lai JL, Calasanz MJ, Cross NC. 2005. Disruption and aberrant expression of HMGA2 as a consequence of diverse chromosomal translocations in myeloid malignancies. Leukemia 19:245-252.
30. Italiano A, Cardot N, Dupre F, Monticelli I, Keslair F, Piche M, Mainguene C, Coindre JM, Pedeutour F. 2007. Gains and complex rearrangements of the 12q13-15 chromosomal region in ordinary lipomas: the "missing link" between lipomas and liposarcomas? Int J Cancer 121:308-315.
31. Jong K, Marchiori E, Van der Vaart A, Chin SF, Carvalho B, Tijssen M, Eijk PP, Van den Ijssel P, Grabsch H, Quirke P, Oudejans JJ, Meijer GA, Caldas C, Ylstra B. 2007. Cross-platform array comparative genomic hybridization meta-analysis separates hematopoietic and mesenchymal from epithelial tumors. Oncogene 26:1499-1506.
33. Cameron HM, Hamperl H, Warambo W. 1974. Leiomyosarcoma of the breast originating from myothelium (myoepithelium). J Pathol 114:89-92.
33. Crissman JD, Wirman JA, Harris A. 1977. Malignant myoepithelioma of the parotid gland. Cancer 40:3042-3049.
34. Fu JF, Hsu JJ, Tang TC, Shih LY. 2003. Identification of CBL, a proto-oncogene at 11q23.3, as a novel MLL fusion partner in a patient with de novo acute myeloid leukemia. Genes Chromosomes Cancer 37:214-219.
35. Buffart TE, Carvalho B, Hopmans E, Brehm V, Kranenbarg EK, Schaaij-Visser TB, Eijk PP, Van Grieken NC, Ylstra B, Van de Velde CJ, Meijer GA. 2007a. Gastric cancers in young and elderly patients show different genomic profiles. J Pathol 211:45-51.
36. Sakamoto I, Ohwada S, Toya H, Togo N, Kashiwabara K, Oyama T, Nakajima T, Ito H, Adachi S, Jigami T, Akiyama T. 2007. Up-regulation of a BCL9-related beta-catenin-binding protein, B9L, in different stages of sporadic colorectal adenoma. Cancer Sci 98:83-87.
37. Myoken Y, Myoken Y, Okamoto T, Sato JD, Kan M, McKeehan WL, Fujihara M, Takada K. 1997. Immunohistochemical localization of fibroblast growth factor-1 (FGF-1), FGF-2 and fibroblast growth factor receptor-1 (FGFR-1) in pleomorphic adenoma of the salivary glands. J Oral Pathol Med 26:17-22.

38. Myoken Y, Myoken Y, Okamoto T, Sato JD, Kan M, McKeehan WL, Nakahara M, Takada K. 1996. Immunohistochemical study of overexpression of fibroblast growth factor-1 (FGF-1), FGF-2, and FGF receptor-1 in human malignant salivary gland tumours. J Pathol 178:429-436.
39. Strehl S, Glatt K, Liu QM, Glatt H, Lalande M. 1998. Characterization of two novel protocadherins (PCDH8 and PCDH9) localized on human chromosome 13 and mouse chromosome 14. Genomics 53:81-89.
40. Franchi A, Gallo O, Bocciolini C, Franchi L, Paglierani M, Santucci M. 1999. Reduced E-cadherin expression correlates with unfavorable prognosis in adenoid cystic carcinoma of salivary glands of the oral cavity. Am J Clin Pathol 111:43-50.
41. Zochbauer-Muller S, Fong KM, Virmani AK, Geradts J, Gazdar AF, Minna JD. 2001. Aberrant promoter methylation of multiple genes in non-small cell lung cancers. Cancer Res 61:249-255.
42. Han SU, Lee HY, Lee JH, Kim WH, Nam H, Kim H, Cho YK, Kim MW, Lee KU. 2005. Modulation of E-cadherin by hepatocyte growth factor induces aggressiveness of gastric carcinoma. Ann Surg 242:676-683.
43. Li L, Yee C, Beavo JA. 1999. CD3- and CD28-dependent induction of PDE7 required for T cell activation. Science 283:848-851.
44. Fan CK. 2006. Phosphodiesterase inhibitors in airways disease. Eur J Pharmacol 533:110-117.
45. Sandros J, Mark J, Happonen RP, Stenman G. 1988. Specificity of 6q- markers and other recurrent deviations in human malignant salivary gland tumors. Anticancer Res 8:637-643.
46. Sandros J, Stenman G, Mark J. 1990. Cytogenetic and molecular observations in human and experimental salivary gland tumors. Cancer Genet Cytogenet 44:153-167.
47. Persson F, Winnes M, Andren Y, Wedell B, Dahlenfors R, Asp J, Mark J, Enlund F, Stenman G. 2007. High-resolution array CGH analysis of salivary gland tumors reveals fusion and amplification of the FGFR1 and PLAG1 genes in ring chromosomes. Oncogene
48. Ntougkos E, Rush R, Scott D, Frankenberg T, Gabra H, Smyth JF, Sellar GC. 2005. The IgLON family in epithelial ovarian cancer: expression profiles and clinicopathologic correlates. Clin Cancer Res 11:5764-5768.
49. Wittmann BM, Wang N, Montano MM. 2003. Identification of a novel inhibitor of breast cell growth that is down-regulated by estrogens and decreased in breast tumors. Cancer Res 63:5151-5158.
50. Schoenmakers EF, Wanschura S, Mols R, Bullerdiek J, Van den BH, Van de Ven WJ. 1995. Recurrent rearrangements in the high mobility group protein gene, HMGI-C, in benign mesenchymal tumours. Nat Genet 10:436-444.
51. Stenman G. 2005. Fusion oncogenes and tumor type specificity-insights from salivary gland tumors. Semin Cancer Biol 15:224-235.
52. Grasser KD. 2003. Chromatin-associated HMGA and HMGB proteins: versatile co-regulators of DNA-dependent processes. Plant Mol Biol 53:281-295.
53. Rao PH, Murty VV, Louie DC, Chaganti RS. 1998. Nonsyntenic amplification of MYC with CDK4 and MDM2 in a malignant mixed tumor of salivary gland. Cancer Genet Cytogenet 105:160-163.
54. Roijer E, Nordkvist A, Strom AK, Ryd W, Behrendt M, Bullerdiek J, Mark J, Stenman G. 2002. Translocation, deletion/amplification, and expression of HMGIC and MDM2 in a carcinoma ex pleomorphic adenoma. Am J Pathol 160:433-440.
55. Collins C, Volik S, Kowbel D, Ginzinger D, Ylstra B, Cloutier T, Hawkins T, Predki P, Martin C, Wernick M, Kuo WL, Alberts A, Gray JW. 2001. Comprehensive genome sequence analysis of a breast cancer amplicon. Genome Res 11:1034-1042.

CHAPTER 5

Deregulated Expression of $p16^{INK4a}$ and p53 Pathway Members in Benign and Malignant Myoepithelial Tumors of the Salivary Glands

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Histopathology 2008, in press.

Chapter 5

ABSTRACT

Myoepithelial salivary gland tumors are uncommon and follow an unpredictable biological course. The aim was to examine their molecular background to acquire a better understanding of their clinical behavior. We investigated protein expression (E2F1, $p16^{INK4a}$, p53, Cyclin D1, Ki-67 and Polycomb group proteins BMI-1, MEL-18, and EZH2) in 49 benign and 30 primary malignant myoepithelial tumors and five histologically benign recurrences by immunohistochemistry and correlated our findings with histopathological characteristics. Benign tumors showed a higher percentage of cells with expression of $p16^{INK4a}$ pathway members [$p16^{INK4a}$ and E2F1 (both $P<0.001$), and Cyclin D1, $P=0.002$] compared with normal salivary gland. Furthermore, malignant tumors expressed in a higher percentage p53 ($P=0.003$) and EZH2 ($P=0.09$). Recurrences displayed more p53+ tumor cells ($P=0.02$) than benign primaries. Amongst the benign tumors, the clear cell type had the highest proliferation fraction ($P=0.05$) and a higher percentage of EZH2 levels was detected in the plasmacytoid cell type ($P=0.002$). Our study is the first to demonstrate that deregulation of the $p16^{INK4a}$ senescence pathway is involved in the development of myoepithelial tumors. We propose that additional inactivation of p53 in malignant primaries and benign recurrences contributes to myoepithelial neoplastic transformation and aggressive tumor growth.

INTRODUCTION

Salivary gland tumors are relatively rare neoplasms that display great variety in their histology and biological behavior. About 80% of these tumors are benign. However, the malignant subtypes often display a poor prognosis due to local recurrences and late metastases.[1]

One of the components of normal salivary gland tissue are myoepithelial cells. These cells exhibit dual epithelial and smooth muscle characteristics.[2] They are contractile, surround ducts and acini and are thought to play a role in the secretion of saliva. Although these myoepithelial cells are frequently recognized in varying proportions in mixed salivary gland tumors, neoplasms composed exclusively of these cells are relatively uncommon (~1% of all salivary gland tumors).[3] Histologically, myoepithelial tumors have varied cell morphology, being spindle-shaped, epithelioid, plasmacytoid, and clear cell, or combinations thereof.[3] It has been proposed that these four cell types may represent different stages in myoepithelial cell differentiation.[4]

Most of these tumors behave in a benign fashion and are designated myoepithelioma (BME). They are typically located in the parotid and minor salivary glands and share cytogenetic and biologic characteristics with pleomorphic adenomas.[5] The malignant counterpart, myoepithelial carcinoma or malignant myoepithelioma (MME), may occasionally arise *de novo*,[6,7] but, particularly in recurrences,[8-10] most often develops in a pre-existing myoepithelioma[11] or in a pleomorphic adenoma.[12-14] The *de novo* form is considered to be more aggressive and to have a higher metastatic potential,[10] however, observations have been inconclusive.[8] Currently, differentiation between benign and malignant myoepitheliomas depends either on mitotic count,[8] presence of invasive growth, cellular polymorphism or tumor necrosis,[15,16] or a combination thereof.

Polycomb group (PcG) proteins are known to control the transcriptional memory of a cell and to play a crucial role in embryonic development, cell cycle regulation and haematopoiesis.[17] Disruption of their mutually exclusive expression pattern has been linked to malignant transformation, loss of differentiation in tumor cells, metastatic behavior and poor prognosis.[18] The mechanism behind aberrant PcG gene expression and cancer might rely on the finding that these proteins

interact with several negative ($p16^{INK4a}$, p53, pRb, p14)[19-22] and positive regulators (E2F1, Cyclin D1)[22] of the cell cycle. Disturbances in PcG protein interaction with these cell cycle controllers can contribute to abnormal cell identity and uncontrolled proliferation.

Cell cycle progression is governed mainly by the $p16^{INK4a}$-Retinoblastoma (pRb) and p53 pathways. $P16^{INK4a}$ induces senescence by negatively regulating the Cyclin D/Cdk complexes and the E2F-dependent transcriptional program.[23] Oncogenic stress can also stabilize p53, leading to cell cycle arrest or the transcriptional activation of pro-apoptotic genes.[24] The core members of these two important pathways ($p16^{INK4a}$, Cyclin D1, E2F1, pRb, and p53, MDMD2, respectively) are the most frequently found targets for mutation, deletion, and epigenetic silencing in all human cancers.[25]

The number of reports dealing with the molecular and biologic disturbances in myoepithelial tumors is limited. DNA content and S-phase fraction have been correlated with aggressive tumor behavior,[26] and expression of the cell cycle proteins p53,[27] Ki-67,[8] c-erbB-2[27] and proliferating cell nuclear antigen[28] has been detected by immunohistochemistry. More recently, cytogenetic analyses concerning chromosomal aberrations in salivary gland tumors have emerged.[29-32] Results of these studies are at times inconclusive and, because of the rarity of myoepithelial tumors, sample sizes have remained relatively small until now.

To shed light on the biologic impact of cell cycle regulatory protein disturbances on salivary myoepithelial tumor initiation and progression, we investigated 49 primary formalin-fixed, paraffin-embedded BMEs and 30 primary MMEs. Furthermore, we included five histologically benign recurrences in our group of neoplasms. By immunohistochemical methods, we detected the expression of $p16^{INK4a}$, p53, Cyclin D1, E2F1 and Ki-67 and expression patterns of the PcG proteins BMI-1, MEL-18, and EZH2. To our knowledge, this is the first paper describing PcG protein expression in salivary gland myoepithelial tumors.

MATERIALS AND METHODS

Sample collection and clinical data

Our series of formalin-fixed, paraffin-embedded myoepithelial tumors was retrieved from two sources: 12 primary BMEs, 23 primary MMEs and two histologically benign recurrences were retrieved from the files of the Salivary Gland Registry at the Department of Oral Pathology (University of Hamburg, Germany). From the archives of the Department of Pathology of the VU University Medical Center (Amsterdam, The Netherlands) 37 primary BMEs, seven primary MMEs and three benign recurrences were collected. Diagnoses were confirmed by experienced salivary gland pathologists (TL and EB for the German and Dutch cases, respectively), and patients were diagnosed with BME or MME from 1975 to 2007. As controls, 17 cases of normal salivary gland tissue samples were used. After haematoxylin and eosin staining, tumor specimens were evaluated by a pathologist (EB) and diagnosed according to cell type. Relevant clinicopathologic factors are summarized in Table 1.

Immunohistochemistry

All tumor samples had been fixed in 4% buffered formalin, processed and embedded in paraffin according to routine procedures. From each tissue block, sections (4 µm) were cut on coated slides and dried overnight at 37 ºC. Sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. Endogenous peroxidase was inhibited for 30 min with 0.3% H_2O_2 in methanol at room temperature and immunohistochemistry was subsequently performed using antibodies and the appropriate detection methods, specified in Table 2. Negative controls were included by substitution of the primary antibody with 1% Bovine Serum Albumin in phosphate-buffered saline. 3-amino-9-ethylcarbazole (Zymed, San Francisco, CA, USA) was used as substrate for the streptavidin and biotinylated horseradish peroxidase complex method and diaminobenzidine for the Envision and Powervision Plus method. Sections were counterstained with haematoxylin, dehydrated and mounted. Photographs were taken

Clinicopathologic parameter	Primary tumors (*n*=79)		Recurrences (*n*=5)
	Myoepitheliomas (*n*=49)	Myoepithelial carcinomas (*n*=30)	
Age, years (range)			
Median	58 (20-82)	55 (32-69)	47 (33-56)
Unknown (*n*)	13	23	2
Gender, *n* (%)			
Male	17 (35)	9 (30)	0
Female	23 (47)	13 (43)	5 (100)
Unknown	9 (18)	8 (27)	0
Histologic cell type, *n* (%)			
Spindle cell	22 (45)	6 (20)	5 (100)
Plasmacytoid	8 (16)	10 (33)	0
Epithelioid	8 (16)	6 (20)	0
Clear	1 (2)	4 (13)	0
Mixed	10 (20)	4 (13)	0
Tumor location, *n* (%)			
Parotid	32 (65)	10 (33)	3 (60)
Submandibular	2 (4)	0	-
Minor salivary glands	5 (10)	1 (3)	-
Parapharynx	2 (4)	1 (3)	-
Unknown	8 (16)	18 (60)	2 (40)

Table 1 Clinicopathologic data of the series of benign and malignant myoepitheliomas.

with a Leica DM 4000 B microscope. Scoring of percentage of immunopositive tumor cells was performed semi-quantitatively by at least two investigators (HV and EB) and included the overall tumor slide. In case of variations in immunopositive cells in the tumor section, the average percentage of expression was taken. For comparison of immuno-expression between normal salivary gland tissue and neoplastic tissue, the expression of markers in the myoepithelial component of the normal salivary gland was taken into account. Differences in intensity of immunoreactivity were not taken into account.

Antibody	Clone	Source	Species	Antigen retrieval	Dilution	Detection method
P53	D07	DAKO	Mouse	Citrate pH=6, MW	1:500	sABC
P16	16P04	Neomarkers	Mouse	Tris/EDTA pH=9, MW	1:200	Envision
E2F1	KH95	Neomarkers	Mouse	Tris/EDTA pH=9, MW	1:250	Powervision Plus
Ki-67	MIB-1	DAKO	Mouse	Citrate pH=6, MW	1:40	Envision
Cyclin D1	DCS-6	Neomarkers	Mouse	Citrate pH=6, AC	1:400	Envision
BMI-1	F6	Upstate	Mouse	Tris/EDTA pH=9, MW	1:500	Powervision Plus
EZH2	11	BD Biosciences	Mouse	Citrate pH=6, AC	1:300	Envision
MEL-18	C-20	Santa Cruz	Goat	Citrate pH=6 AC	1:400	sABC-BT

Table 2 Antibodies and staining conditions for immunohistochemistry. EDTA, ethylenediamine tetraaceticacid; sABC, streptavidin and biotinylated horseradish peroxidase complex; MW, microwave; AC, autoclave; o/n, overnight; BT, biotinylated tyramine.

Statistical analysis

Because of the non-normal distribution of protein expression levels, statistical evaluation was performed using non-parametric tests. Comparison between protein expression levels in tumor and normal tissue was carried out with the use of the Mann-Whitney *U* test. When multiple independent groups were compared, the Kruskal-Wallis *H* test was employed. Associations between protein expression and histopathologic parameters were performed by Fischer's exact probability test. A two-sided *P*-value of ≤0.05 was considered to be statistically significant. Statistical analyses were performed with the use of the Statistical Package for the Social Sciences, version 15.0.1 (SPSS Inc., Chicago, IL, USA).

RESULTS

Significantly increased expression of $p16^{INK4a}$ pathway members in myoepitheliomas

In normal salivary gland tissue (*n*=17), expression of all the cell cycle proteins was low (Figure 1), both in ductal as well as in acinar and myoepithelial cells: median Ki-67 of 1% (range, 1-15), E2F1 of 3% (range, 1-5), Cyclin D1 of 1% (range, 1-10), $p16^{INK4a}$ of 3% (range, 1-10) and p53 of 3% (range, 1-10). In the BMEs (*n*=49) however, expression of the investigated p16 pathway members was significantly increased (Figure 2a-c): median p16 of 10% (*P*<0.001), median Cyclin D1 of 10% (*P*=0.002) and median E2F1 of 10% (*P*<0.001).

The PcG proteins displayed mutually exclusive expression patterns in salivary gland tissue, with PRC1 members being abundantly present (Figure 3): median BMI-1 level of 95% (range, 80-100), MEL-18 of 40% (range, 10-95) and EZH2 of 1% (range, 1-5). Statistical analysis showed a significant increase in the BMEs for BMI-1 (median, 100%, range, 50-100; *P*=0.05) and MEL-18 (median, 90%; range, 0-100; *P*=0.002) proteins. However, since no elevation of EZH2 expression was observed (median, 0%; range, 0-5; Figure 2d), the mutually exclusive expression pattern of this protein group was maintained.

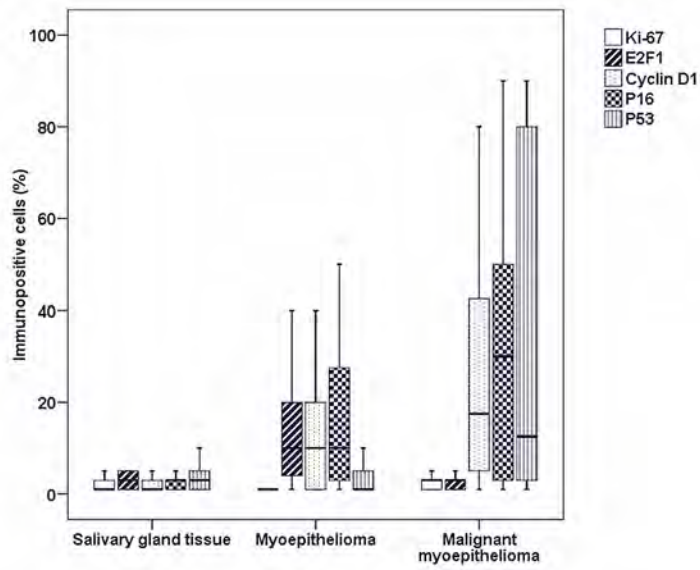

Figure 1 Expression of the cell cycle proteins in normal and neoplastic salivary gland tissues. Thick black line represents median number of immunopositive cells. Area of boxplot corresponds to the interquartile range (IQR), displaying 50% of the middle values. Cyclin D1 ($p=0.002$), $p16^{INK4a}$ ($p<0.001$), and E2F1 ($p<0.001$) are significantly increased in benign myoepitheliomas (BMEs) compared with normal salivary gland tissue. In malignant myoepitheliomas (MMEs), in addition to an increase in Cyclin D1 and $p16^{INK4a}$ (both $p<0.001$), elevated p53 was observed ($p=0.003$). A significantly lower percentage of cells expressed E2F1 in MMEs than in BMEs ($p<0.001$).

Members of both pathways (i.e. $p16^{INK4a}$ and p53) are deregulated in malignant myoepithelioma

The significant deregulation in the expression of p16 pathway proteins as observed in BMEs was also detected in the MMEs ($n=30$) when compared with normal salivary gland tissue: median Cyclin D1 level was 15% (range, 1-80; $P<0.001$) and $p16^{INK4a}$ was 35% (range, 1-90; $P<0.001$). Furthermore, significantly elevated p53 expression was detected in these malignant tumors (median, 8%; range, 1-90; $P=0.003$; Figures 1 and 4a-c).

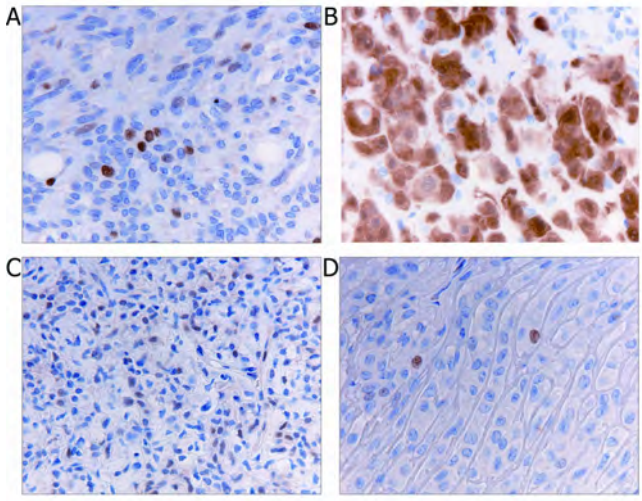

Figure 2 Representative patterns of immunoreactivity in benign myoepitheliomas of **(a)** E2F1, **(b)** $p16^{INK4a}$, **(c)** Cyclin D1, and **d)** EZH2.

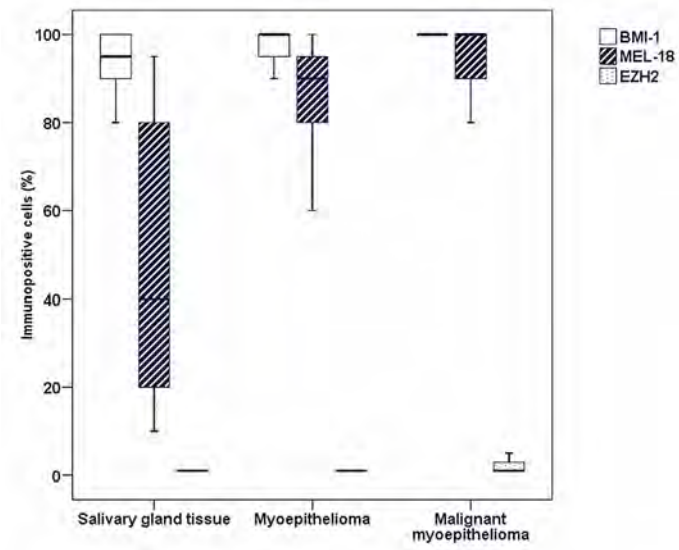

Figure 3 Expression patterns of the Polycomb group (PcG) proteins in normal and neoplastic salivary gland tissues. Thick black line represents median number of immunopositive cells. Area of box plot corresponds to the interquartile range (IQR), displaying 50% of the middle values. In benign myoepitheliomas (BMEs) compared with normal tissue, significantly increased expression of BMI-1 and MEL-18 was observed (P=0.05 and P=0.002, respectively). A significantly higher percentage of cells expressed EZH2 in malignant myoepitheliomas (MMEs) than in BMEs (P=0.005).

Comparison of BMEs and MMEs revealed a significant increase of p53 (median, 1%; range, 0-50 vs. 8%; range, 0-90, respectively;

P<0.001), Cyclin D1 (median, 10%; range, 0-70 vs. 15%; range, 1-80, respectively; *P*=0.02), Ki-67 (median, 1%; range, 0-8 vs. 3%; range, 0-50, respectively; *P*=0.002), EZH2 (median, 0%; range, 0-5 vs. 1%; range, 0-90, respectively; *P*=0.005) and MEL-18 (median, 90%; range 0-100 vs. 100%; range, 1-100, respectively; *P*=0.008) in the malignant tumors. Interestingly, the amount of E2F1 immunopositive cells was significantly lower in MMEs compared with BMEs (median, 3%; range, 0-50 vs. 10%; range, 1-50, respectively; *P*<0.001).

PRC1 members BMI-1 and MEL-18 displayed in MMEs about the same expression levels and significance as in BMEs (median, 100%; range, 80-100; *P*=0.03 and median, 100%; range, 1-100; *P*<0.001, respectively). In contrast, the EZH2 protein tended to be more variably expressed in these malignant tumors (median, 1%; range, 1-90; *P*=0.09) compared with normal salivary gland tissue (Figures 3 and 4d).

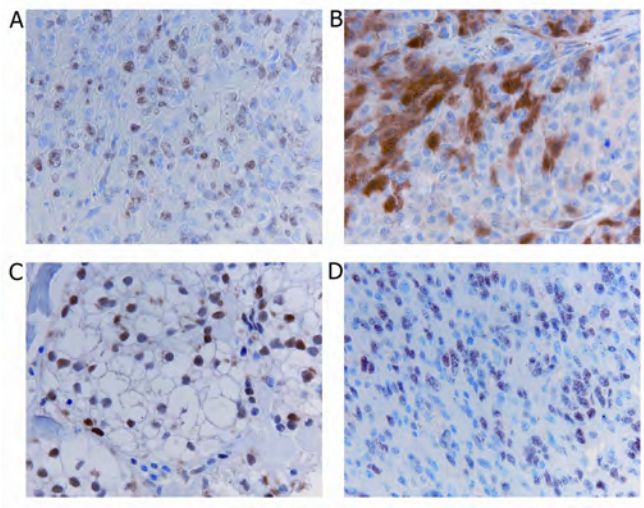

Figure 4 Representative patterns of immunoreactivity in malignant myoepitheliomas of **(a)** Ki-67, **(b)** $p16^{INK4a}$, **(c)** p53 and **(d)** EZH2.

Elevated p53 expression in recurrences compared with primary tumors

In addition to the primary lesions, we separately analyzed five histologically benign recurrences originating from benign primaries. When comparing protein expression levels in the primary BMEs (*n*=49) and in the recurrences, significantly elevated levels of p53 were

observed in the recurrent lesions (median, 1%; range, 0-50 vs. 15%; range, 0-70, respectively; *P*=0.02).

Correlation of cytological subtypes with protein expression levels

Correlation of cell type (plasmacytoid, epithelioid, spindle cell, clear cell and mixed) with protein expression levels revealed significantly higher MEL-18 levels in MMEs of the clear cell type compared with the other cell types (all cases 100% vs. median 100%; range, 1-100, respectively; *P*=0.05). The clear cell type in BMEs showed an increased proliferative rate when compared with other cell types (5% vs. median 1%; range, 0-8, respectively; *P*=0.05). Also, significantly higher EZH2 expression was observed in the plasmacytoid cell type in BMEs than in other cytological subtypes (median 2%; range, 0-3 vs. 0%; range, 0-8, respectively; *P*=0.002).

DISCUSSION

Even though salivary gland tumors displaying exclusively myoepithelial differentiation have been recognized since 1943,[33] Dardick *et al.* were the real pioneers in clarifying and expanding the histopathologic characteristics of these lesions.[34,35] They recognized that myoepithelial cells are functionally and phenotypically complex and that their morphologic plasticity is mirrored in the tumors arising from them. Currently, even though diagnosis of myoepitheliomas might be less complex, molecular disturbances underlying their development are not well characterized. In this study, our aim was to investigate expression patterns of several cell cycle proteins (E2F1, $p16^{INK4a}$, p53, Cyclin D1 and Ki-67) together with PcG proteins BMI-1, MEL-18 and EZH2 in these neoplasms by immunohistochemistry.

When comparing BMEs with normal salivary gland tissue, significant up-regulation of proteins involved in the $p16^{INK4a}$ pathway was observed (proteins $p16^{INK4a}$ and Cyclin D1). Significantly increased levels of transcription factor E2F1, as detected here, have also been described in pleomorphic adenomas.[36] Overexpression of E2F1 together with $p16^{INK4a}$ accumulation is a frequent event in various neoplasms as a response to Rb protein deletion or mutation.[36-38] It would be interesting to

investigate whether altered Rb levels indeed contribute to the deregulation of the senescence pathway observed in these salivary gland lesions. The low median expression rates of Ki-67 and p53 in these tumors are in concordance with earlier observations in benign salivary gland neoplasms.[8,27,39]

Interestingly, in addition to the significant increase in $p16^{INK4a}$ pathway members, MMEs also displayed significant up-regulation of p53. Also in the benign recurrent tumors, a significant increase in p53 expression was detected. These results suggest that p53 plays an important part in the malignant progression of myoepithelial cells and aggressive tumor behavior. There is controversy, however, concerning p53's role in myoepithelial salivary gland tumors. On one hand, several reports have described high p53 levels in malignant neoplasms;[8,40] on the other hand, Rosa *et al.* did not find high accumulations of p53 in several malignant salivary gland tumors with myoepithelial differentiation.[27] We believe, based on our current and previous data,[50] that high p53 expression is in fact correlated with malignant progression and possibly also with aggressive behavior in myoepithelial tumors.

Only one paper so far has addressed the expression of Rb pathway members in a MME.[36] In agreement with our findings, the authors detected a significant increase of Cyclin D1 and $p16^{INK4a}$ proteins compared with normal salivary gland tissue. Interestingly, they also found decreased expression of E2F1 (i.e. 0%) in this tumor compared with normal salivary gland tissue. Mouse knockout models have shown that *E2F1* possesses tumor suppressive characteristics in a tissue- and time-specific manner.[41] Besides several types of tumors, dysplasia of the salivary glands was observed in these *E2F1*-/- mice. When growth arrest was induced in a human submandibular gland cell line, *E2F1* overexpression diverted the cells into an apoptotic pathway.[42] Considering the fact that the tumor-suppressive effects of E2F1 require the presence of a functional p53 pathway,[43] it is tempting to speculate that malignant progression in myoepithelial tumors is, at least in part, prevented by a functional p53 apoptosis pathway in combination with tumor-suppressive effects of E2F1.

The mutually exclusive expression pattern of the two PcG complexes is maintained in these myoepithelial tumors. However, EZH2 expression increased significantly in MMEs compared with BMEs. In adenoid cystic

carcinoma, a salivary gland tumor with myoepithelial differentiation, high EZH2 expression has also been observed.[50]

As mentioned before, it has been suggested that the four myoepithelial cell types represent different stages in myoepithelial cell differentiation.[4,8] Since plasmacytoid cells do not exhibit myogenous differentiation,[44,45] these cells are considered to be poorly differentiated. At the other end of the spectrum are the spindle cells, which express smooth muscle markers consistently. In concordance with our results, Ogawa *et al.* have detected the highest proliferative activity in the well-differentiated spindle and clear cell types.[28] Interestingly, increased EZH2 expression has been related to loss of differentiation in tumor cells.[46] We detected a similar expression profile of this PcG protein in our malignant tumors.

Histological features have thus far failed to predict the biological behavior of myoepithelial tumors reliably. Most tumors displaying perineural growth, cytological atypia, high mitotic rate and necrosis behave aggressively, but the same histological features have also been reported in indolent tumors.[8,47] One explanation for this might be that their biological potential and aggressiveness are influenced by their underlying biochemical secretory properties. Myoepithelial cells possess an intrinsic capacity to exhibit anti-invasive characteristics by secretion of proteinase inhibitors[48] and an anti-angiogenic phenotype by expressing active angiogenic inhibitors.[49] Modifications of these biochemical features, rather than histological appearance, have been suggested to play the determining role in the behavior of these carcinomas.[9]

Taken together, hallmarks of human cancer, such as alterations in the $p16^{INK4a}$ senescence and p53 apoptosis pathways, seem to be major events in these myoepithelial tumors. Although our study has not unraveled the precise levels of deregulation in these pathways, our observations by immunohistochemistry allow us to speculate that malignant transformation in myoepithelial cells requires, in addition to $p16^{INK4a}$ pathway deregulation, inactivation of p53. Also, based on previous results by others and our current results, it would be interesting to address the possible tumor-suppressive effects of E2F1 in salivary gland tissue in future studies.

Acknowledgements

The authors thank E. C. Strang and C. B. W. Strijbos for obtaining the patient data. W. de Jong, E. Aghazadeh and M. Koning are acknowledged for their technical assistance. This work was financially supported by the Interuniversity Research School (IOT), Amsterdam, The Netherlands.

Chapter 5

REFERENCES

1. Speight PM, Barrett AW. Salivary gland tumors. Oral Dis. 2002;8;229-240.
2. Dardick I. A role for electron microscopy in salivary gland neoplasms. Ultrastruct. Pathol. 1985;9;151-161.
3. Barnes L, Eveson JW, Reichart P, Sidransky D.World Health Organization Classification of Tumors: Head and Neck Tumors Lyon: IARCPress, 2005.
4. Da Silveira EJ, Pereira AL, Fontora MC, De Souza LB, De Almeida FR. Myoepithelioma of minor salivary gland--an immunohistochemical analysis of four cases. Rev. Bras. Otorrinolaringol. (Engl. Ed) 2006;72;528-532.
5. El Naggar AK, Lovell M, Callender DL, Ordonez NG, Killary AM. Cytogenetic analysis of a primary salivary gland myoepithelioma. Cancer Genet. Cytogenet. 1999;113;49-53.
6. Crissman JD, Wirman JA, Harris A. Malignant myoepithelioma of the parotid gland. Cancer 1977;40;3042-3049.
7. Dean A, Sierra R, Alamillos FJ et al. Malignant myoepithelioma of the salivary glands: clinicopathological and immunohistochemical features. Br. J Oral Maxillofac. Surg. 1999;37;64-66.
8. Nagao T, Sugano I, Ishida Y et al. Salivary gland malignant myoepithelioma: a clinicopathologic and immunohistochemical study of ten cases. Cancer 1998;83;1292-1299.
9. Savera AT, Sloman A, Huvos AG, Klimstra DS. Myoepithelial carcinoma of the salivary glands: a clinicopathologic study of 25 patients. Am. J Surg. Pathol. 2000;24;761-774.
10. Di Palma S, Guzzo M. Malignant myoepithelioma of salivary glands: clinicopathological features of ten cases. Virchows Arch. A Pathol. Anat. Histopathol. 1993;423;389-396.
11. Alos L, Cardesa A, Bombi JA, Mallofre C, Cuchi A, Traserra J. Myoepithelial tumors of salivary glands: a clinicopathologic, immunohistochemical, ultrastructural, and flow-cytometric study. Semin. Diagn. Pathol. 1996;13;138-147.
12. Singh R, Cawson RA. Malignant myoepithelial carcinoma (myoepithelioma) arising in a pleomorphic adenoma of the parotid gland. An immunohistochemical study and review of the literature. Oral Surg. Oral Med. Oral Pathol. 1988;66;65-70.
13. McCluggage WG, Primrose WJ, Toner PG. Myoepithelial carcinoma (malignant myoepithelioma) of the parotid gland arising in a pleomorphic adenoma. J Clin. Pathol. 1998;51;552-556.
14. Karatzanis AD, Drivas EI, Giannikaki ES, Lachanas VA, Hatziioannou JK, Velegrakis GA. Malignant myoepithelioma arising from recurrent pleomorphic adenoma of the soft palate. Auris Nasus Larynx 2005;32;435-437.
15. Dardick I, Cavell S, Boivin M et al. Salivary gland myoepithelioma variants. Histological, ultrastructural, and immunocytological features. Virchows Arch. A Pathol. Anat. Histopathol. 1989;416;25-42.
16. Ellis GL, Auclair PL. Tumors of the Salivary Glands. Atlas of tumor pathology, 3rd series, fasicle 17. Washington D.C.: Armed Forces Institute of Pathology. 1996.
17. Satijn DP, Otte AP. Polycomb group protein complexes: do different complexes regulate distinct target genes? Biochim. Biophys. Acta 1999;1447;1-16.
18. Raaphorst FM. Deregulated expression of Polycomb-group oncogenes in human malignant lymphomas and epithelial tumors. Hum. Mol. Genet. 2005;14 Suppl 1;R93-R100.
19. Bracken AP, Kleine-Kohlbrecher D, Dietrich N et al. The Polycomb group proteins bind throughout the INK4A-ARF locus and are disassociated in senescent cells. Genes Dev. 2007;21;525-530.
20. Jacobs JJ, Kieboom K, Marino S, DePinho RA, Van Lohuizen M. The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. Nature 1999;397;164-168.

21. Tang X, Milyavsky M, Shats I, Erez N, Goldfinger N, Rotter V. Activated p53 suppresses the histone methyltransferase EZH2 gene. Oncogene 2004;23;5759-5769.
22. Bracken AP, Pasini D, Capra M, Prosperini E, Colli E, Helin K. EZH2 is downstream of the pRB-E2F pathway, essential for proliferation and amplified in cancer. EMBO J 2003;22;5323-5335.
23. Michalides RJ. Cell cycle regulators: mechanisms and their role in aetiology, prognosis, and treatment of cancer. J Clin. Pathol. 1999;52;555-568.
24. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000;408;307-310.
25. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100;57-70.
26. El Naggar A, Batsakis JG, Luna MA, Goepfert H, Tortoledo ME. DNA content and proliferative activity of myoepitheliomas. J Laryngol. Otol. 1989;103;1192-1197.
27. Rosa JC, Felix A, Fonseca I, Soares J. Immunoexpression of c-erbB-2 and p53 in benign and malignant salivary neoplasms with myoepithelial differentiation. J Clin. Pathol. 1997;50;661-663.
28. Ogawa I, Miyauchi M, Takata T, Vuhahula E, Ijuhin N, Nikai H. Proliferative activity of salivary gland pleomorphic adenomas and myoepitheliomas as evaluated by the proliferating cell nuclear antigen (PCNA) labeling index (LI). J Oral Pathol. Med. 1993;22;447-450.
29. Hungermann D, Roeser K, Buerger H, Jakel T, Loning T, Herbst H. Relative paucity of gross genetic alterations in myoepitheliomas and myoepithelial carcinomas of salivary glands. J Pathol. 2002;198;487-494.
30. Vékony H, Ylstra B, Wilting SM et al. DNA Copy Number Gains at Loci of Growth Factors and Their Receptors in Salivary Gland Adenoid Cystic Carcinoma. Clin. Cancer Res. 2007;13;3133-3139.
31. Martins C, Fonseca I, Roque L, Ribeiro C, Soares J. Cytogenetic similarities between two types of salivary gland carcinomas: adenoid cystic carcinoma and polymorphous low-grade adenocarcinoma. Cancer Genet. Cytogenet. 2001;128;130-136.
32. Martins C, Fonseca I, Felix A, Roque L, Soares J. Benign salivary gland tumors: a cytogenetic study of 21 cases. J Surg. Oncol. 1995;60;232-237.
33. Sheldon WH. So-called mixed tumors of the salivary glands. Arch. Pathol. 1943;35;1-20.
34. Dardick I, Thomas MJ, Van Nostrand AW. Myoepithelioma--new concepts of histology and classification: a light and electron microscopic study. Ultrastruct. Pathol. 1989;13;187-224.
35. Dardick I. Myoepithelioma: definitions and diagnostic criteria. Ultrastruct. Pathol. 1995;19;335-345.
36. Etges A, Nunes FD, Ribeiro KC, Araujo VC. Immunohistochemical expression of retinoblastoma pathway proteins in normal salivary glands and in salivary gland tumors. Oral Oncol. 2004;40;326-331.
37. Kaye FJ. RB and cyclin dependent kinase pathways: defining a distinction between RB and p16 loss in lung cancer. Oncogene 2002;21;6908-6914.
38. Sabah M, Cummins R, Leader M, Kay E. Aberrant expression of the Rb pathway proteins in soft tissue sarcomas. Appl. Immunohistochem. Mol. Morphol. 2006;14;397-403.
39. Alves FA, Pires FR, De Almeida OP, Lopes MA, Kowalski LP. PCNA, Ki-67 and p53 expressions in submandibular salivary gland tumors. Int. J. Oral Maxillofac. Surg. 2004;33;593-597.
40. Yamamoto Y, Kishimoto Y, Wistuba II et al. DNA analysis at p53 locus in carcinomas arising from pleomorphic adenomas of salivary glands: comparison of molecular study and p53 immunostaining. Pathol. Int. 1998;48;265-272.
41. Yamasaki L, Jacks T, Bronson R, Goillot E, Harlow E, Dyson NJ. Tumor induction and tissue atrophy in mice lacking E2F-1. Cell 1996;85;537-548.
42. Lillibridge CD, O'Connell BC. In human salivary gland cells, overexpression of E2F1 overcomes an interferon-gamma- and tumor necrosis factor-alpha-induced growth arrest but does not result in complete mitosis. J Cell Physiol 1997;172;343-350.

43. Russell JL, Weaks RL, Berton TR, Johnson DG. E2F1 suppresses skin carcinogenesis via the ARF-p53 pathway. Oncogene 2006;25;867-876.
44. Takai Y, Dardick I, Mackay A, Burford-Mason A, Mori M. Diagnostic criteria for neoplastic myoepithelial cells in pleomorphic adenomas and myoepitheliomas. Immunocytochemical detection of muscle-specific actin, cytokeratin 14, vimentin, and glial fibrillary acidic protein. Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod. 1995;79;330-341.
45. De Araujo VC, Carvalho YR, De Araujo NS. Actin versus vimentin in myoepithelial cells of salivary gland tumors. A comparative study. Oral Surg. Oral Med. Oral Pathol. 1994;77;387-391.
46. Raaphorst FM, Meijer CJ, Fieret E et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia. 2003;5;481-488.
47. Dardick I. Malignant myoepithelioma of parotid salivary gland. Ultrastruct. Pathol. 1985;9;163-168.
48. Sternlicht MD, Kedeshian P, Shao ZM, Safarians S, Barsky SH. The human myoepithelial cell is a natural tumor suppressor. Clin. Cancer Res. 1997;3;1949-1958.
49. Nguyen M, Lee MC, Wang JL et al. The human myoepithelial cell displays a multifaceted anti-angiogenic phenotype. Oncogene 2000;19;3449-3459.
50. Vékony H, Raaphorst FM, Otte AP, Van Lohuizen M, Leemans CR, Van der Waal I, Bloemena E. High expression of Polycomb Group protein EZH2 predicts poor survival in salivary gland adenoid cystic carcinoma. J. Clin. Pathol. 2008; 6; 744-749.

CHAPTER 6

Salivary Gland Carcinosarcoma: Oligonucleotide Array CGH Reveals Similar Genomic Profiles in Epithelial and Mesenchymal Components

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Oral Oncology 2008, in press.

ABSTRACT

In this study, we present a case of parotid gland de novo carcinosarcoma. Salivary gland carcinosarcoma (or true malignant mixed tumor) is a rare biphasic neoplasm, composed of both malignant epithelial and malignant mesenchymal components. It is yet unclear whether these two phenotypes occur by collision of two independent tumors or if they are of clonal origin. To analyze the clonality of the different morphologic tumor components, oligonucleotide microarray-based comparative genomic hybridization (oaCGH) was performed on the carcinoma and the sarcoma entity separately. This technique enables a high-resolution, genome-wide overview of the chromosomal alterations in the distinct tumor elements. Analysis of both fractions showed a high number of DNA copy number changes. Losses were more prevalent than gains (82 and 49, respectively). The carcinomatous element displayed more chromosomal aberrations than the sarcomatous component. Specific amplifications of MUC20 (in mesenchymal element) and BMI-1 (in both elements) loci were observed. Overall homology between the two genomic profiles was 75%. DNA copy number profiles of the epithelial and mesenchymal components in this salivary gland carcinosarcoma displayed extensive overlap, indicating a monoclonal origin. Since losses are shared to a larger extent than gains, they seem to be more essential for initial oncogenic events. Furthermore, specific amplifications of a mucin and a Polycomb group gene imply these proteins in the tumorigenesis of carcinosarcomas.

INTRODUCTION

The first report on a carcinosarcoma in a salivary gland was published in 1967.[1] Due to its rarity, current knowledge about this malignant mixed tumor is still limited. Carcinosarcomas are biphasic tumors exhibiting both carcinomatous and sarcomatous elements and since the 2005 WHO classification of salivary gland tumors, they are recognized as a distinct entity (true malignant mixed tumors).[2,3] Most cases consist of poorly differentiated adenocarcinoma (NOS) or salivary duct carcinoma in combination with a variable mesenchymal component, most often chondrosarcoma or osteosarcoma. These malignant neoplasms are thought to arise either in a pleiomorphic adenoma (PA) or, if no remnants of a benign tumors is present, de novo. Generally, carcinosarcomas display frequent local recurrences and metastases resulting in a poor 5-year survival.[4,5]

A long standing debate exists as to whether the two morphologically different elements in carcinosarcomas arise from a common precursor cell or whether they arise separately and represent a collision tumor.[6] In the former case, the two histologically different components would result from a dichotomous differentiation pattern of a common stem cell or by transdifferentiation (metaplasia) from one cell type to another. In other organs, a monoclonal origin of the two cellular components of carcinosarcomas has already been indicated.[7-9] Very few molecular studies have addressed this question in the salivary glands,[10,11] but those that have, support a single pluripotent stem cell divergence hypothesis.[4,9] Additionally, recent findings have implicated the myoepithelial cell to underlie the development of both the sarcomatous and carcinomatous component in carcinosarcomas in general.[4,9,12,13]

Comparative genomic hybridization (CGH) allows the measurement of copy number increases and decreases (i.e. gains and losses, respectively) at specific chromosomal regions. Specific gains and losses have not only opened the way to the detection of a series of cancer-related genes, but also to clinical implications, including differential diagnosis, and prognosis of disease progression.[14] Furthermore, since chromosomal aberrations seem to harbor a certain tumor-specificity, copy number profiles can be used for classification.[15] Oligonucleotide microarray-based CGH (oaCGH) is a new application that provides high-

resolution, genome-covering information on DNA copy number changes, even for formalin-fixed archival material.[16]

The focus of this study was to apply oaCGH on a de novo carcinosarcoma of the parotid gland to identify the specific genomic profiles of the two cellular components.

MATERIALS AND METHODS

Patient

A 59-year-old woman presented with a mass in the right parotid gland. The tumor was resected and macroscopic examination of the cut surface revealed a 3.5 cm large nodule, with central necrosis (Figure 1). Histological evaluation revealed that the tumor consisted of two components, an adenocarcinoma and a sarcoma composed of spindle-shaped cells (Figure 2a). The epithelial part of the tumor consisted of a cohesive groups of cells with large, polymorphic nuclei and eosinophilic cytoplasm. Focally, luminal formation was present. The tumor was diagnosed as a carcinosarcoma.

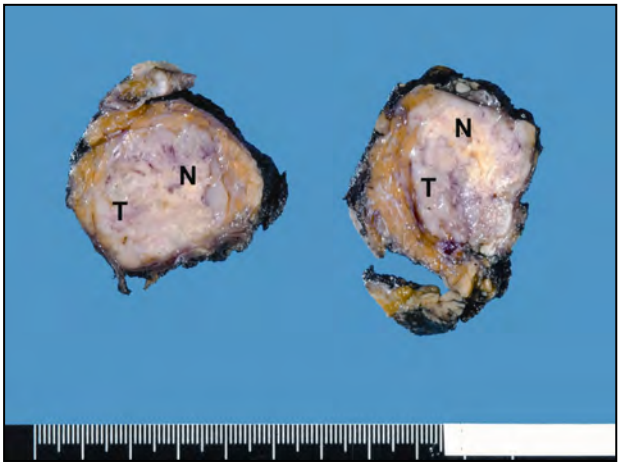


Figure 1 Overview of the cross-section of the resected parotid gland carcinosarcoma, depicting the pinkish tumor area at the periphery **(T)**, together with central necrosis **(N)**.

Immunohistochemistry

To determine the differentiation patterns of tumor cells in both components, immunostaining with specific markers was performed. First, the obtained specimen was fixed in 4% buffered formalin,

processed, and embedded in paraffin according to routine procedures. From each tissue block, sections (4 µm) were cut on coated slides and dried overnight at 37ºC. Sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. Then, immunohistochemical staining with CAM 5.2 (BD Biosciences, Breda, The Netherlands) was performed at 1:10 with the Ventana Medical Systems-NexES IHC Staining Module. Vimentin (in-house antibody, clone V9) and P63 (clone 4A4+Y4A3; Immunologic, Duiven, The Netherlands) were performed with the BOND-maX Vision Biosystems module at 1:100 000 and 1:800, respectively. Calponin (clone CALP1; Dako, Glostrup, Denmark) was used at 1:750 with the BOND-maX Vision Biosystems module. AE1/AE3 (Chemicon International Inc., Temecula, CA, USA) was diluted at 1:100 and staining was performed in the Ventana Medical Systems-NexES IHC Staining Module. S100 (Dako, Glostrup, Denmark), a polyclonal antibody, was used at 1:5 000 in the BOND-maX Vision Biosystems module.

DNA extraction

Of each paraffin block, haematoxylin and eosin (H&E) stainings were reviewed to assure that at least 70% tumor cells were present before sectioning and DNA extraction. Genomic DNA was extracted from three 10 µm thick formalin-fixed, paraffin-embedded (FFPE) tissue sections from both components separately. After deparaffinating with xylene and rehydrating with decreasing concentrations of ethanol (100% - 96% - 70%), the sections were haematoxylin stained and tumor areas were macrodissected. DNA extraction was performed according to standard protocols of proteinase K (1mg/ml) digestion overnight, followed by phenol-chloroform extraction. DNA quality was assessed by isothermal amplification, as described previously.[17]

Oligonucleotide microarray-based comparative genomic hybridization (oaCGH)

To explore the genomic profiles of the two distinct cellular components of the carcinosarcoma, we employed a 4x44k array CGH platform including 45,214 60-mer oligonucleotides. This allowed us a genome-wide survey and molecular karyotyping of genomic aberrations with an

average resolution of 75 kb.[18] Labeling of tumor and reference DNA was performed using the ENZO BioArray™ CGH Labeling System (Farmingdale, NY, USA). Genomic tumor DNA (500 ng) was labeled with Cy3 and mixed with a normal human reference pool of ten healthy individuals (labeled with Cy5) prior to hybridization on a 4x44K Agilent slide (Amstelveen, The Netherlands) using the Agilent hybridization oven (G2545A) overnight at 20 rpm. The oligo CGH-microarray slide was scanned using a DNA microarray scanner G2565AB (Agilent Technologies Netherlands B.V., Amstelveen, the Netherlands).Washing, scanning and feature selection (Feature extraction software v.9.1) were furthermore performed using standard Agilent procedures. Data presented include all spots printed on the arrays and are available via GEO series accession number GSE12958.

Data processing

In order to determine exact breakpoints in the generated array CGH profiles, we segmented the obtained $\log_2$ ratios by DNAcopy.[19] Sex chromosomes were discarded from the analysis, since all tumor samples were hybridized to a pool of reference DNA of the opposite gender. In the downstream analysis, only clones with no missing values were included. Segmented $\log_2$ ratios were converted to four levels of categorized data (i.e. losses, normals, gains, and amplifications) by CGHCall,[20] implemented using the statistical software environment R [R Development Core Team (2006)].

RESULTS

Immunohistochemistry

Immunohistochemical staining showed that epithelial cells were positive for CAM5.2 (Figure 2b). Spindle cells demonstrated immunoreactivity to vimentin and p63 (Figure 2c and 2d). Furthermore, AE1/AE3 and S100 immunopositivity was detected in the carcinomatous and the sarcomatous component, respectively (data not shown). Interestingly, calponin staining was evident in both elements (data not shown), supporting a common stem cell hypothesis.

Oligonucleotide array CGH

Chromosomal aberrations in the epithelial and mesenchymal component of the tumor were analyzed by oaCGH. The carcinosarcoma showed a high number of alterations in both cellular entities (Table 1). A total of 26 and 23 gains were detected in the carcinoma and in the sarcoma, respectively. Losses were even more frequent, namely 47 in the epithelial and 35 in the mesenchymal component. As a whole, the epithelial element harbored more genomic events than the mesenchymal part (73 versus 58, respectively). Six high-level amplifications were found in the sarcoma opposed to three in the carcinoma (Table 2). Regarding four amplified regions, a corresponding gain was detected in the other component. A specific amplification of the BMI-1 locus was observed in both tumor elements. An overview of the chromosomal profiles in the epithelial and mesenchymal part of the tumor is depicted in Figure 3.

Next, we determined the level of similarity in the chromosomal profiles. Clones in both components were compared and the amount of oligonucleotides exhibiting homologous copy numbers (i.e. loss, gain, normal or amplification) in both components was divided by the total amount of clones (i.e. 40488). Analysis showed substantial overlap between the two genomes, since 75% of all copy numbers was concordant. If we only took the chromosomal imbalances into account, 34% of the DNA copy numbers showed homologous losses, gains, or amplifications.

Table 1 Overview of all chromosomal aberrations as detected by oligo CGH in the mesenchymal and epithelial component of the carcinosarcoma. Bold cytobands represent genomic differences between the two cellular elements. All other chromosomal aberrations are detected in both cellular elements.

	Sarcoma	**Carcinoma**
Gains	1q23.3, 1q42.12-q44 **2p13.1** **4q11-q12** **5q31.3** 6q22.31 7p22.3-p22.1, **7p14.1-p13, 7q11.23, 7q22.1** 12p13.31, 12p11.21-p11.1, 12q13.13, **12q24.21-q24.31** 14q21.1 16p13.3, 16p11.2 17q11.1-q11.2, 17q21.2-q21.31, 17q25.1-q25.3 19p13.3-p13.11, 19q13.12 21q22.3	1q21.1-q23.3, 1q31.2-q44 **2p16.2-p16.1, 2q32.1** **3p26.3-p24.1** 6q22.31-q22.32 7p22.1, **7q34**, **7q35-q36.3** **8p12-p11.21, 8q13.1-q23.1, 8q23.3-q24.23, 8q24.3** **9q34.3** **10p15.1, 10p12.2, 10q22.2** 12p13.33-p11.1, 12q13.13, **12q21.2-q21.31, 12q24.11-q24.12** 16p13.3-p11.2 17q11.1-q25.3 19p13.13, 19q13.11-q13.12 21q22.11
Losses	1p31.2-q21.1 **2p22.3** 3p23-p21.31, 3p21.1-p12.1, 3q13.31-q25.31 4p16.3-q11, **4q35.2** 5p15.33-q31.3, 5q31.3-q35.3 6p25.3-p22.2, 6p21.31-p12 9p24.3-p21.1, 9p13.2-q33.3 10q11.23-q26.3 11p15.5-p15.4, 11p15.3-q11, 11q12.1-q12.2, 11q13.2-q13.3, 11q13.4-q13.5, 11q21-q23.3, 11q24.1-q25 12q12, 12q14.1-q14.3 13q12.11-q34 14q11.2, 14q12-q21, 14q21.1-q32.33 17p13.3- p13.1, 17p13.1-p11.2, 17p11.2-q11.1 18p11.32-q23 19q13.12-q13.43 20p13-p11.21 21p11.1-q11.2 22q11.1-q11.21	1p31.2-p22.3, 1p22.2-q21.1 **3p24.1,** 3p23-p12.1, 3q13.31-q21.3, 3q21.3-q25.31 4p16.1-p11 5p15.33-p15.31, 5p15.2-p13.3, 5p13.2-q15, 5q15-q21.3, 5q21.3-q35.2, 5q35.3 6p25.3-p21.33 6p21.32-p21.2 7p11.2-p11.1 9p24.3-q13, **9q34.13** 10q11.23-q21.2, 10q21.3-q22.1, 10q22.1-q22.2, 10q22.2-q26.3 11p15.5-q13.1, 11q13.2-q23.3, 11q23.3-q24.1, 11q24.1-q25 12q12, **12q13.11-q13.12**, 12q13.3-q14.1, 12q14.1-q14.3, 13q12.11-q32.3, 13q32.3-q34 14q11.2, 14q11.2-q32.31, 14q32.32, 14q32.32-q32.33 16q22.1 17p13.3-p12, 17p11.2-p11.1 18p11.32-q23 19q13.12-q13.33, 19q13.33-q13.43 20p13-p11.21, 20p11.1-q13.13, **20q13.13-q13.33** 21p11.1-q11.2 22q11.1-q11.21

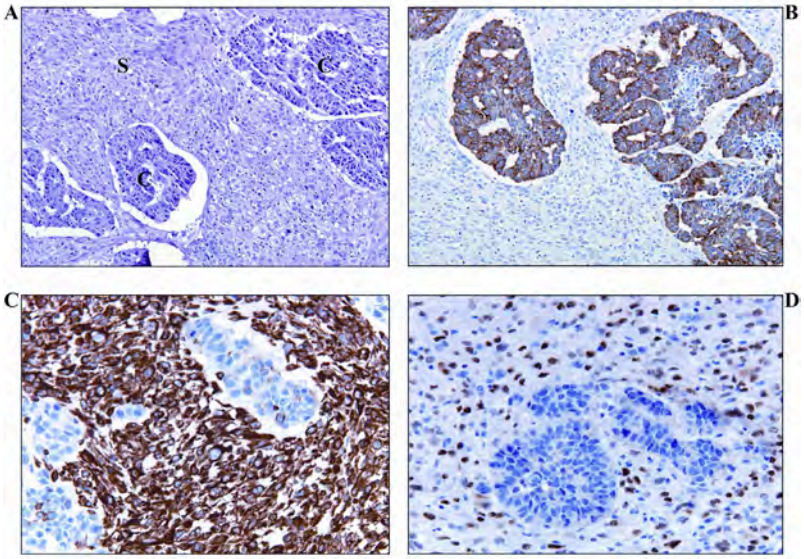


Figure 2 Immunohistochemical evaluation of the carcinosarcoma. **A.** Haematoxylin-eosin staining of the tumor, showing small nests of carcinoma (C) and large areas of sarcoma (S) (200x). **B.** Immunostaining with CAM5.2 is only detected in the carcinomatous islands (200x). **C**. Strong vimentin and **D.** p63 staining of the mesenchymal cells (both 400x).

	Cytoband	Size (Mbps)	Candidate genes
Sarcoma	1q23.3*	0.11	*KCNJ9, IGSF8, ATP1A2*
	3q29	0.003	*MUC20*
	10p12.2*	0.001	*BMI-1*
	16q23.1	1.17	*CLEC3A*
	19q13.11-q13.12*	1.39	*MLL4*
	21q22.11*	0.11	*KRTAP8-1, KRTAP21-2*
Carcinoma	4q11-q21.1*	25.03	*PDGFRA, EREG, MUC7*
	10p12.2*	0.001	*BMI-1*
	12q13.11	0.33	*SLC38A1*

Table 2 Overview of the high-level amplifications as detected by oligo CGH in the sarcomatous and the carcinomatous components. Potentially interesting genes located in the amplified region are depicted. (*) clones also gained/amplified in other tumor component; abbreviation: Mbps – mega-basepares

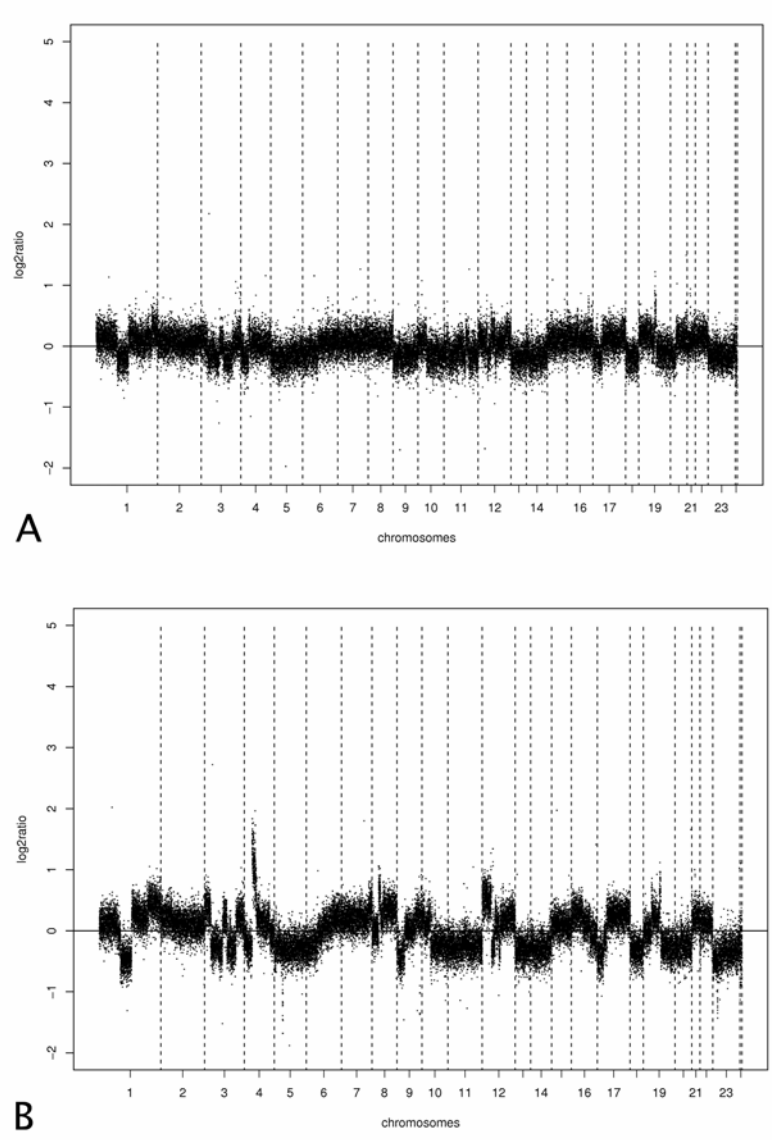


Figure 3 Genome-wide frequency plots of DNA copy number variations identified by oaCGH in (**A)** the sarcomatous and (**B)** in the carcinomatous components. Log_2 ratios for each of the oligonucleotide clones are plotted along the chromosomal axis.

DISCUSSION

In carcinosarcomas (or true malignant mixed tumors), both cellular elements histologically show malignancy.[3] These high-grade tumors display aggressive biological behavior and a high mortality rate.[6,21,22] Consequently, more knowledge about their background might facilitate treatment modalities. An important question concerns their histogenesis: do the distinct cellular entities reflect two independent neoplastic processes or are they genetically related? Although not entirely conclusive yet, most evidence supports the monoclonality hypothesis.[6,10,12,23,24]

In the present study, we examined the chromosomal profiles of the carcinomatous and the sarcomatous element separately by oaCGH. If carcinosarcomas indeed would have a unicellular origin, a substantial overlap would be apparent in the patterns of chromosomal alterations of both cellular elements.

First, we screened for genomic aberrations that are possibly associated with either component. In accordance with earlier studies, both phenotypically different elements showed a high number of chromosomal aberrations[23,24] and losses of DNA were more frequent than gains.[11] In our tumor, the carcinoma demonstrated a higher amount of chromosomal instability. Analysis by our department and others has detected more genetic hits in the mesenchymal component.[10,11,23] Frequent chromosomal alterations reported previously in both components of carcinosarcomas include gains of chromosome 7 and 8q, and losses of chromosome 10 and 9p. Furthermore, epithelial cells often display a loss at 13q and mesenchymal cells typically gain regions at 12q.[11,23,25,26] With the exception of the 8q gain, which was in our case restricted to the carcinomatous component, all common aberrations were also detected in our study.

Since two amplifications in our tumor specifically target a single gene, they might be relevant for the tumorigenesis of carcinosarcomas. MUC20, located at 3q29 and amplified in the mesenchymal component, belongs to the mucin family of proteins. These gene products are known to be overexpressed in various types of cancer[27,28] and to correlate with poor outcome.[29] Interestingly, the 10p12.2 high-level amplification was shared by both components and specifically contained the proto-

oncogene BMI-1. This protein belongs to the Polycomb group proteins, which have been detected to be deregulated in several neoplasms,[30] including salivary gland tumors.[31] We did not observe amplification at the Cyclin D1 locus (at 11q12-q13) as was found in a case of vulvar carcinosarcoma,[26] probably due to tissue dependent oncogenesis.

Secondly, we addressed the question of clonality. Although the monoclonal hypothesis is widely favored over the collision theory, prior reports on conformity between the genomic profiles of the two elements differ from only one alteration shared[11] to 91% similarity.[23] Fowler *et al.* tested loss-of-heterozygosity (LOH) rates at loci of known tumor suppressors in carcinosarcomas and they found a 73% agreement in the mutational profiles of the two components.[10] We found a 75% homology between the DNA copy numbers of the two cellular components. When taking only the chromosomal aberrations into account, 34% of all gains, losses and amplifications overlapped. Thus, our data are consistent with previous reports on highly shared genomic profiles.

In conclusion, the extensively overlapping genomic profiles of the two cellular components strongly indicate that the epithelial and mesenchymal elements of carcinosarcomas have evolved from a single common precursor cell. This is further underlined by the finding that the myoepithelial marker calponin was immunopositive in both tumor components. Since genomic deletions in this tumor are shared to a greater extent than gains, it seems that losses of tumor suppressor genes are more essential for the histologic evolution of carcinosarcomas than gains/amplifications of oncogenes. Also, since amplifications of MUC20 and BMI-1 loci were highly specific, it would be interesting to explore the roles of mucins and Polycomb group proteins in the development of carcinosarcomas.

Acknowledgments

We are indebted to A. Mrsic, P.P. Eijk, and W. de Jong for technical assistance. This work was supported financially by the Interuniversity Research School (IOT), Amsterdam, the Netherlands.

REFERENCES

1. King OH, Jr. Carcinosarcoma of accessory salivary gland. First report of a case. Oral Surg Oral Med Oral Pathol 1967;23(5):651-9.
2. Gnepp DR. Malignant mixed tumors of the salivary glands: a review. Pathol Annu 1993;28 (Pt 1):279-328.
3. Barnes L, Eveson JW, Reichart P, Sidransky D, editors. World Health Organization Classification of Tumours: Pathology and Genetics of Head and Neck Tumours. Lyon: IARCPress, 2005. p. 244.
4. Gotte K, Riedel F, Coy JF, Spahn V, Hormann K. Salivary gland carcinosarcoma: immunohistochemical, molecular genetic and electron microscopic findings. Oral Oncol 2000;36(4):360-4.
5. Pang PC, To EW, Tsang WM, Liu TL. Carcinosarcoma (malignant mixed tumor) of the parotid gland: a case report. J Oral Maxillofac Surg 2001;59(5):583-7.
6. Stephen J, Batsakis JG, Luna MA, Von der HU, Byers RM. True malignant mixed tumors (carcinosarcoma) of salivary glands. Oral Surg Oral Med Oral Pathol 1986;61(6):597-602.
7. Rossi G, Cavazza A, Sturm N, Migaldi M, Facciolongo N, Longo L, et al. Pulmonary carcinomas with pleomorphic, sarcomatoid, or sarcomatous elements: a clinicopathologic and immunohistochemical study of 75 cases. Am J Surg Pathol 2003;27(3):311-24.
8. Matsumoto T, Fujii H, Arakawa A, Yamasaki S, Sonoue H, Hattori K, et al. Loss of heterozygosity analysis shows monoclonal evolution with frequent genetic progression and divergence in esophageal carcinosarcoma. Hum Pathol 2004;35(3):322-7.
9. Thompson L, Chang B, Barsky SH. Monoclonal origins of malignant mixed tumors (carcinosarcomas). Evidence for a divergent histogenesis. Am J Surg Pathol 1996;20(3):277-85.
10. Fowler MH, Fowler J, Ducatman B, Barnes L, Hunt JL. Malignant mixed tumors of the salivary gland: a study of loss of heterozygosity in tumor suppressor genes. Mod Pathol 2006;19(3):350-5.
11. Volker HU, Muhlmeier G, Maier H, Kraft K, Muller-Hermelink HK, Zettl A. True malignant mixed tumour (carcinosarcoma) of submandibular gland--a rare neoplasm of monoclonal origin? Histopathology 2007;50(6):795-8.
12. Staffieri C, Marioni G, Ferraro SM, Marino F, Staffieri A. Carcinosarcoma de novo of the parotid gland. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2007;104(2):e35-e40.
13. Gandour-Edwards RF, Donald PJ, Vogt PJ, Munn R, Min KW. Carcinosarcoma (malignant mixed tumor) of the parotid: report of a case with a pure rhabdomyosarcoma component. Head Neck 1994;16(4):379-82.
14. Pinkel D, Albertson DG. Array comparative genomic hybridization and its applications in cancer. Nat Genet 2005;37(Suppl):S11-S7.
15. Jong K, Marchiori E, Van der Vaart A, Chin SF, Carvalho B, Tijssen M, et al. Cross-platform array comparative genomic hybridization meta-analysis separates hematopoietic and mesenchymal from epithelial tumors. Oncogene 2007;26(10):1499-1506.
16. Van den Ijssel P, Ylstra B. Oligonucleotide array comparative genomic hybridization. Methods Mol Biol 2007;396:207-22.
17. Buffart TE, Tijssen M, Krugers T, Carvalho B, Smeets SJ, Brakenhoff RH, et al. DNA quality assessment for array CGH by isothermal whole genome amplification. Cell Oncol 2007;29(4):351-59.
18. Coe BP, Ylstra B, Carvalho B, Meijer GA, MacAulay C, Lam WL. Resolving the resolution of array CGH. Genomics 2007;89(5):647-53.
19. Olshen AB, Venkatraman ES, Lucito R, Wigler M. Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics 2004;5(4):557-72.

20. Van de Wiel MA, Kim KI, Vosse SJ, Van Wieringen WN, Wilting SM, Ylstra B. CGHcall: calling aberrations for array CGH tumor profiles. Bioinformatics 2007;23(7):892-4.
21. Tortoledo ME, Luna MA, Batsakis JG. Carcinomas ex pleomorphic adenoma and malignant mixed tumors. Histomorphologic indexes. Arch Otolaryngol 1984;110(3):172-6.
22. Ellis GL, Auclair PL. Tumors of the Salivary Glands. Atlas of tumor pathology, 3rd series, fasicle 17. Washington D.C.: Armed Forces Institute of Pathology, 1996. p. 259-60.
23. Torenbeek R, Hermsen MA, Meijer GA, Baak JP, Meijer CJ. Analysis by comparative genomic hybridization of epithelial and spindle cell components in sarcomatoid carcinoma and carcinosarcoma: histogenetic aspects. J Pathol 1999;189(3):338-43.
24. Teixeira MR, Qvist H, Bohler PJ, Pandis N, Heim S. Cytogenetic analysis shows that carcinosarcomas of the breast are of monoclonal origin. Genes Chromosomes Cancer 1998;22(2):145-51.
25. Gronau S, Menz CK, Melzner I, Hautmann R, Moller P, Barth TF. Immunohistomorphologic and molecular cytogenetic analysis of a carcinosarcoma of the urinary bladder. Virchows Arch 2002;440(4):436-40.
26. Adam P, Zettl A, Zollner U, Dietl J, Muller-Hermelink HK, Eck M. Metastasizing vulvar carcinosarcoma with squamous carcinomatous and leiomyosarcomatous differentiation: genetic evidence of clonal origin. Hum Pathol 2005;36(10):1143-7.
27. Vlad AM, Diaconu I, Gantt KR. MUC1 in endometriosis and ovarian cancer. Immunol Res 2006;36(1-3):229-36.
28. Singh AP, Chaturvedi P, Batra SK. Emerging roles of MUC4 in cancer: a novel target for diagnosis and therapy. Cancer Res 2007;67(2):433-6.
29. Woenckhaus M, Merk J, Stoehr R, Schaeper F, Gaumann A, Wiebe K et al. Prognostic value of FHIT, CTNNB1, and MUC1 expression in non-small cell lung cancer. Hum Pathol 2008;39(1):126-36.
30. Raaphorst FM. Deregulated expression of Polycomb-group oncogenes in human malignant lymphomas and epithelial tumors. Hum Mol Genet 2005;14(Suppl 1):R93-R100.
31. Vékony H, Raaphorst FM, Otte AP, Van Lohuizen M, Leemans CR, Van der Waal I et al. High expression of Polycomb group protein EZH2 predicts poor survival in salivary gland adenoid cystic carcinoma. J Clin Pathol 2008; 6: 744-749.

CHAPTER 7

General Discussion

Malignant progression in myoepithelial salivary gland tumors correlates with accumulating disturbances in essential cell cycle pathways

The expression profiles of cell cycle pathway members in normal salivary gland tissue reveal that expression of p53, $p16^{INK4a}$, Cyclin D1, and E2F1 is marginal, as is also reported for other normal, healthy tissues. However, during malignant transformation, levels of these regulatory proteins are increased (**Chapters 3 and 5**).

Since evolution of cancer is a progressive and cumulative process, it is not surprising that the degree of protein disturbances in the tumors we investigated (i.e. BME, MME, and ACC) parallels their biological and clinical aggressiveness. Of these, ACC has the highest level of deregulation in its cell cycle pathways. Accordingly, this tumor displays the most aggressive biological course among the three types, by having high recurrence and metastasis rates, and resulting in poor patient outcome.

Based on our results, alterations in the Rb-$p16^{INK4a}$ pathway can be considered as an early event in myoepithelial salivary gland tumor development. The benign myoepitheliomas harbor deregulated activation of Rb pathway members without p53 expression. For transition towards more aggressive tumor behavior, another oncogenic 'hit' is needed, which is provided by deregulation of the p53 pathway. This hypothesis is endorsed in our histologically benign recurred myoepitheliomas, which have a tendency to express p53.

Previous studies support this theory by also implicating accumulation of the p53 protein in the malignant transformation of salivary gland tumors. In contrast to pleiomorphic adenomas, (myoepithelial) carcinomas arising in these benign tumors tend to express p53 or display LOH at the p53 locus.[1-4] It has been suggested that development of these malignant tumors may be a result of inactivation of p53. In ACC and malignant myoepithelioma, elevated p53 expression is detected at later stages of tumor progression and correlates with shorter survival.[5,6]

It is important to note that cell cycle pathways act in an extremely complex molecular network. Although our study has focused on several

of its key players, we have investigated them only on a protein level. By exploring the expression of more cell cycle pathway members, preferably also at mRNA level, genetic alterations in salivary gland tumors can be unraveled more precisely and put into context of their relevant networks or pathways.

The elucidate the functional role of potentially interesting cell cycle and PcG proteins in salivary gland development, *in vitro* studies are needed. The cell line ACC3 was derived in 1995 from a solid ACC subtype of the parotid gland and maintains phenotypic characteristics of primary disease.[7-9] The establishment of experimental systems with gene-specific siRNA constructs to knockdown candidate proteins in combination with, for example, cell invasion assays would verify whether our proposed proteins indeed contribute to myoepithelial salivary gland tumor initiation and progression.

A combination of Cyclin D1, EZH2 and $p16^{INK4a}$ expression is able to define an ACC patient group with favorable prognosis

Given the variability and complexity of salivary gland tumors, current prognostic markers are not able to reliably predict their biological behavior. Histologically benign tumors can sometimes display an aggressive clinical course with multiple recurrences and distant metastases. It is thus of foremost importance to identify at an earliest time point possible which patient groups have an increased likelihood of developing progressive disease or poor survival. To assist in the prognostication of myoepithelial salivary gland neoplasms, we have determined the clinical significance of the investigated biomarkers.

In patients with ACC ($n=21$; **Chapter 3**), we were able to identify a subgroup with a highly favorable outcome based on the combined expression profiles of Cyclin D1, EZH2 and $p16^{INK4a}$. Correlation of protein expression with clinical data of the patients revealed that high p16 expression is predicative of the development of recurrent disease ($p=0.05$), and that low expression of Cyclin D1 correlates with metastasis ($p=0.01$). Furthermore, tumors exhibiting ≥40% EZH2 expressing cells displayed a significantly shorter survival time ($p=0.04$). Sequentially combining the expression levels of Cyclin D1, EZH2 and

$p16^{INK4a}$, we achieved to define a patient group with long term overall survival (Figure 1). Patients with high Cyclin D1 expression (≥10%) combined with high EZH2 expression (≥40%) all died of the disease within 7 years (*n*=5). Patients with high Cyclin D1 expression, low EZH2 expression and low p16 levels (<40%) all survived, with a mean follow-up time of 70 months (*n*=10; range, 30-128 months).

Notably, the clinical relevance of our survival algorithm needs to be validated in an additional and preferably larger patient group of which extensive follow-up data is available.

High levels of the PcG protein EZH2 correlate with aggressive behavior of myoepithelial salivary gland tumors

Over the last decade, the link between abnormal PcG protein expression and the initiation and progression of several human and murine cancers has been the highlight of numerous studies. As two PcG proteins have been identified as targets for therapeutic intervention recently, they are the subject of considerable interest: BMI-1 has been implicated in the maintenance of self-renewal of normal and cancer stem cells[10] and elevated levels of EZH2 seem to correlate in almost every human tumor with aggressive behavior and adverse clinical course,[11,12] designating EZH2 as a prognostic marker.

Our investigations are the first in the field of salivary gland tumor research dealing with PcG protein expression patterns (**Chapters 3 and 5**). As in the case of other solid human cancers, EZH2 was expressed in the clinically most aggressive tumors. The solid histologic pattern in ACC, considered as a high-grade neoplasm with poor differentiation, displayed the highest level of EZH2 expression. Since EZH2 deregulation has been correlated with invasive tumor behavior and poor differentiation, our data concur with previous findings.[13] Moreover, patients expressing ≥40% EZH2 protein in their tumor cells showed a significantly shorter survival time than patients with less immunoreactivity.

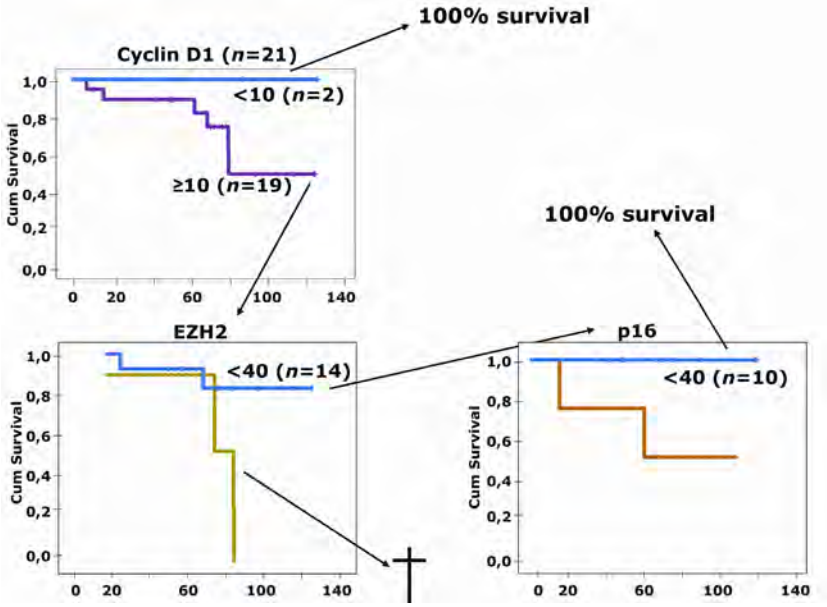


Figure 1 Survival algorithm based on Cyclin D1, EZH2 and p16 expression levels for 21 adenoid cystic carcinoma (ACC) patients (see text for details).

Our group of myoepithelial carcinomas also displayed increased EZH2 levels compared to their benign counterparts, supporting the connection between this PcG protein and malignancy. The plasmacytoid cellular type, considered as the most poorly differentiated myoepithelial cell, expressed the highest level of EZH2 protein. Future *in vitro* studies, as proposed for the cell cycle proteins, should shed some light on the functional involvement of EZH2 in salivary gland tumorigenesis. If these additional experiments agree with our hypothesis, this protein might constitute an exciting novel target for salivary gland cancer therapy. EZH2 is known to trimethylate histone tails, to influence DNA methylation by direct interaction with DNA methyltransferases and its deregulated expression plays an important role in aberrant cancer gene silencing.[14-16] Since epigenetic changes are potentially reversible, they make attractive targets for therapeutic intervention. Initial approaches have recently uncovered, that reactivation of PRC2 target genes selectively induces apoptosis in breast cancer cells.[17]

Genomic aberrations in myoepithelial salivary gland tumors point to growth factors and growth factor receptors to be involved in their initiation and progression

By genome-wide measurement of DNA copy number variations in 15 BMEs, 12 MMEs, and 18 ACCs (**Chapters 2 and 4**), frequent copy number increases were found at sites of several growth factors (specifically FGFs and PDGFβ) and growth factor receptors (specifically FGFRs, PDGRFβ, and EGFR).

Several lines of research have previously implicated growth factors in salivary gland tumorigenesis. First, immunohistochemical techniques have detected overexpression of the mitogens FGF1, FGF2, and their receptor FGFR1 in malignant and benign salivary gland tumors.[18,19] Secondly, malignant transformation of salivary adenocarcinoma has been shown to coincide with *de novo* expression of FGFR1 and FGFR4 concomitantly with keratinocyte growth factor receptor (KGFR) loss.[20] Third, EGFR overexpression has been observed in ACC immunohistochemically,[21,22] and *in vivo* and *in vitro* administration of an EGFR inhibitor, alone or in combination with chemotherapy, led to tumor growth inhibition, induction of apoptosis and prevention of lung metastases.[22] In addition to their mitogenic activity, FGFs have been shown to increase the production of proteases in tumor cells *in vitro*, thereby providing a potential means to promote tissue invasiveness.[23] In corroboration with this, our subgroup of aggressive ACCs displayed significantly more DNA copy number gains at FGF loci compared to indolent tumors.

Based on earlier findings and our array CGH results, we hypothesize that FGFs have a significant role in myoepithelial salivary gland tumorigenesis. Furthermore, the presence of both receptor and ligand suggests the possible existence of an autocrine stimulation loop to induce tumor cell proliferation.

To provide further evidence, immunohistochemical stainings against the receptors and their ligands should to be performed in future experiments.

Chapter 7

Salivary gland tumors arise according to the "pluripotent stem cell histogenesis concept"

In an attempt to shed light on the complex morphologic appearance of salivary gland tumors, two different theories about their histogenesis have been developed. The "pluripotent stem cell theory" states that both the normal salivary gland unit and the different tumor types are the result of differentiation of pluripotent precursor cells.[24] A problem in the normal differentiation process is then responsible for the neoplastic transformation of cells. The earlier in differentiation the problem occurs, the more undifferentiated and "high grade" the resulting tumor type. The "multicellular theory" favors the transformation of the entire ducto-acinar unit and thus requires the various differentiated cells to become "dedifferentiated", deranged in their growth pattern, to result in the combination of the different components observed in the numerous types of salivary gland tumors.

To investigate whether we could provide data supporting one of these histogenetic concepts, we analyzed the genomic profiles of a rare, biphasic malignant salivary gland tumor, a carcinosarcoma (**Chapter 6**). This tumor consists of two clearly separate epithelial and mesenchymal elements, which are both malignant. The two opposing hypotheses, i.e. multi- or monoclonality, have been advanced to explain the origin of these neoplasms. These true malignant mixed tumors have been observed at diverse anatomic sites, such as the lungs,[25] the esophagus,[26] the breast,[27] the vulva[28] and the uterus,[29] and a monoclonal origin of the two components in these organ systems has been indicated. Until now, very few genetic studies have concentrated on salivary gland carcinosarcomas.[30-32]

By detecting a considerable overlap (75%) in the genomic profiles of the distinct morphologic elements in this carcinosarcoma, we provide additional evidence that this biphasic tumor develops from a common pluripotent stem cell. At a certain stage during the process of carcinogenesis, the primitive stem cell, not yet committed to either epithelial or mesenchymal differentiation, probably gives rise to both morphologically distinct cell populations. According to the "multicellular theory", the two components would result from dedifferentiation of two phenotypically distinct cell types and would thus not share the oncogenic

alterations leading to transformation. We propose, that based on our and previous other findings,[33] salivary gland histo- and tumorigenesis occurs via the differentiation of a single stem cell.

Tumors with myoepithelial differentiation are characterized by a unique pattern of DNA copy number variations

Myoepithelial differentiation implies both epithelial and mesenchymal (i.e. smooth muscle) features in one cell. To investigate whether the myoepithelial nature of BMEs, ACCs, and MMEs is expressed in their chromosomal signature, we performed cluster analysis of array CGH data of these tumors with other well-characterized epithelial and mesenchymal neoplasms. Therefore, colon carcinomas (n=11), gastric carcinomas (n=19), mamma adenocarcinomas (n=5), lung adenocarcinomas (n=4), glioblastomas (n=8), soft tissue leiomyosarcomas (n=10), and soft tissue sarcomas (n=4), together with ACCs (n=17), BMEs (n=15), and MMEs (n=11) were subjected to a modified version of WECCA cluster analysis (described in **Chapter 5**). Dual channel BAC array CGH data from the primary epithelial (n=38) and brain tumors was collected from the publicly available GEO database (www.ncbi.nlm.nih.gov/geo/). Additionally, the mesenchymal tumors (n=14) were subjected to array CGH analysis.

We performed cluster analysis based on call-probabilities for each feature on the array (a probability of a loss, of no-aberration, and of a gain). The use of call probabilities in downstream analyses prevents the loss of information associated with the use of calls. Additionally, genomically adjacent features not separated by a breakpoint have the same call probability signature over the samples. The use of these call probabilities in our unsupervised WECCA analysis gives a more subtle picture of the similarities and differences between the samples.

As shown in Supplementary Figure S1, clustering was based to some extent on the number of aberrations detected in the neoplasms, since tumors with relatively few DNA copy number changes were grouped together. Although the epithelial and mesenchymal tumors did not separate completely, several well-defined clusters were formed based on their genomic profiles.

With this method, we were able to identify three major clusters in this group of 103 primary tumors (Figure 2). Cluster I contained mostly leiomyosarcomas (8/10) and glioblastomas (6/8) and these neoplasms were grouped together based mostly on losses of chromosomes 10 and 13, and a gain of chromosome 7. Since glioblastomas have been shown to express mesenchymal stem-like properties,[34] their joined grouping with the leiomyosarcomas is not unexpected.

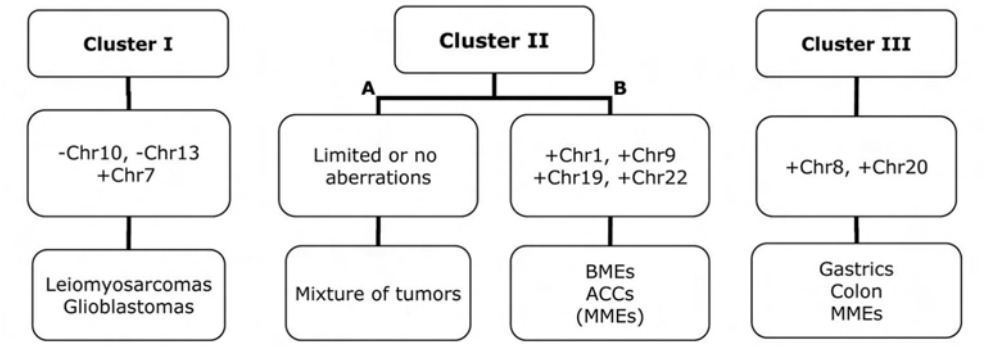


Figure 2 Diagram depicting the three clusters and subclusters created by WECCA analysis based on call-probabilities. The most distinctive chromosomal changes, together with the main tumor types in the individual clusters are summarized.

Overall, cluster II harbored tumors with no or a limited number of chromosomal changes. Not surprisingly, a large part of this group comprised a mixture of neoplasms expressing epithelial, mesenchymal, and myoepithelial features (Figure S1; A). However, our myoepithelial tumors clustered separately (B), characterized primarily by gains on chromosomes 1, 9, 19 and 22. Twelve of the 17 ACCs, 8/15 of the BMEs and 3/11 of the MMEs shared these unique DNA copy number patterns. Cluster III consisted of tumors with epithelial origin (14/19 gastric and 6/11 colon cancers), together with 6/11 MMEs. Apparently, MMEs exhibit genomic features (i.e. gains of chromosomes 8 and 20) typical for epithelial tumors.

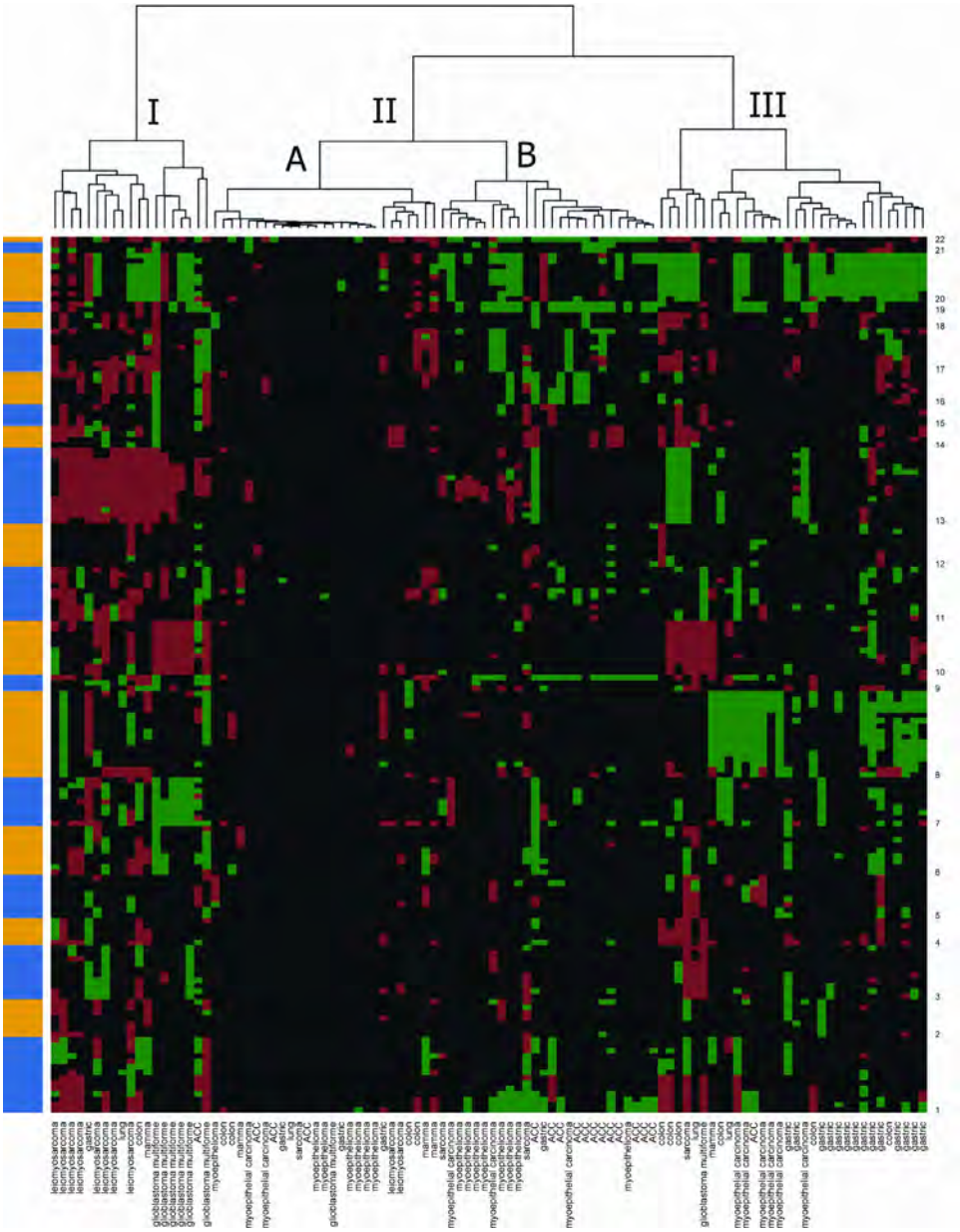
A recent cross-platform meta-analysis applied to array CGH data of 373 primary tumors was able to separate neoplasms with epithelial and mesenchymal origin based on their DNA copy number patterns, implicating that chromosomal changes that drive tumorigenesis are shared not only by tissue but also by embryonic origin.[35] Although myoepithelial cells exhibit both epithelial and mesenchymal features, tumors with myoepithelial differentiation appear to have unique genomic changes, not shared by cancers of epithelial or mesenchymal origin. The oncogenic mechanisms underlying myoepithelial tumor development are evidently different from other tumors types and it would be interesting to investigate the chromosomal gains on chromosomes 1, 9, 19 and 22 in more detail. These distinctive DNA copy number increases seem to be important for myoepithelial tumor development and probably harbor oncogenes driving myoepithelial tumorigenesis. Interestingly, recurrent chromosomal alterations specific for epithelial tumors (i.e. gains of chromosomes 8 and 20) were also detected in 55% of our MMEs. Frequent gains on chromosome 8 have been reported previously in myoepithelial carcinomas[36] and our array CGH data also detected this genomic region to contribute to malignant progression (**Chapter 4**). We propose that this chromosomal area specifically harbors oncogenes involved in the tumorigenesis of myoepithelial carcinomas and should be investigated in more detail in future studies.

Chapter 7

References

1. Yamamoto,Y, Kishimoto,Y, Virmani,AK et al. Mutations associated with carcinomas arising from pleomorphic adenomas of the salivary glands. Hum Pathol, 1996. 27(8):782-786.
2. McCluggage,WG, Primrose,WJ, and Toner,PG. Myoepithelial carcinoma (malignant myoepithelioma) of the parotid gland arising in a pleomorphic adenoma. J Clin Pathol, 1998. 51(7):552-556.
3. El Naggar,AK, Callender,D, Coombes,MM et al. Molecular genetic alterations in carcinoma ex-pleomorphic adenoma: a putative progression model? Genes Chromosomes Cancer, 2000. 27(2):162-168.
4. El Naggar,AK, Hurr,K, Kagan,J et al. Genotypic alterations in benign and malignant salivary gland tumors: histogenetic and clinical implications. Am J Surg Pathol, 1997. 21(6):691-697.
5. Zhu,QR, White,FH, and Tipoe,GL. p53 oncoprotein accumulation in adenoid cystic carcinoma of parotid and palatine salivary glands. Pathology, 1997. 29(2):154-158.
6. Papadaki,H, Finkelstein,SD, Kounelis,S et al. The role of p53 mutation and protein expression in primary and recurrent adenoid cystic carcinoma. Hum Pathol, 1996. 27(6):567-572.
7. He,R, Zhou,X, and Xia,W. [Observations of cellular differentiation behaviour in adenoid cystic carcinoma of human salivary gland in vitro]. Zhonghua Kou Qiang Yi Xue Za Zhi, 1996. 31(3):150-152.
8. Frierson,HF, Jr., el Naggar,AK, Welsh,JB et al. Large scale molecular analysis identifies genes with altered expression in salivary adenoid cystic carcinoma. Am J Pathol, 2002. 161(4):1315-1323.
9. Cheng,J, Irie,T, Munakata,R et al. Biosynthesis of basement membrane molecules by salivary adenoid cystic carcinoma cells: an immunofluorescence and confocal microscopic study. Virchows Arch, 1995. 426(6):577-586.
10. Raaphorst,FM. Self-renewal of hematopoietic and leukemic stem cells: a central role for the Polycomb-group gene Bmi-1. Trends Immunol, 2003. 24(10):522-524.
11. Tonini,T, D'Andrilli,G, Fucito,A et al. Importance of Ezh2 polycomb protein in tumorigenesis process interfering with the pathway of growth suppressive key elements. J Cell Physiol, 2008. 214(2):295-300.
12. Bachmann,IM, Halvorsen,OJ, Collett,K et al. EZH2 expression is associated with high proliferation rate and aggressive tumor subgroups in cutaneous melanoma and cancers of the endometrium, prostate, and breast. J Clin Oncol, 2006. 24(2):268-273.
13. Raaphorst,FM, Meijer,CJ, Fieret,E et al. Poorly differentiated breast carcinoma is associated with increased expression of the human polycomb group EZH2 gene. Neoplasia, 2003. 5(6):481-488.
14. Cao,R and Zhang,Y. The functions of E(Z)/EZH2-mediated methylation of lysine 27 in histone H3. Curr Opin Genet Dev, 2004. 14(2):155-164.
15. Vire,E, Brenner,C, Deplus,R et al. The Polycomb group protein EZH2 directly controls DNA methylation. Nature, 2005.
16. Martin,C, Cao,R, and Zhang,Y. Substrate preferences of the EZH2 histone methyltransferase complex. J Biol Chem, 2006. 281(13):8365-8370.
17. Tan,J, Yang,X, Zhuang,L et al. Pharmacologic disruption of Polycomb-repressive complex 2-mediated gene repression selectively induces apoptosis in cancer cells. Genes Dev, 2007. 21(9):1050-1063.
18. Myoken,Y, Myoken,Y, Okamoto,T et al. Immunohistochemical study of overexpression of fibroblast growth factor-1 (FGF-1), FGF-2, and FGF receptor-1 in human malignant salivary gland tumours. J Pathol, 1996. 178(4):429-436.

19. Myoken,Y, Myoken,Y, Okamoto,T et al. Immunohistochemical localization of fibroblast growth factor-1 (FGF-1), FGF-2 and fibroblast growth factor receptor-1 (FGFR-1) in pleomorphic adenoma of the salivary glands. J Oral Pathol Med, 1997. 26(1):17-22.
20. Zhang,Y, Wang,H, Toratani,S et al. Growth inhibition by keratinocyte growth factor receptor of human salivary adenocarcinoma cells through induction of differentiation and apoptosis. Proc Natl Acad Sci U S A, 2001. 98(20):11336-11340.
21. Vered,M, Braunstein,E, and Buchner,A. Immunohistochemical study of epidermal growth factor receptor in adenoid cystic carcinoma of salivary gland origin. Head Neck, 2002. 24(7):632-636.
22. Younes,MN, Park,YW, Yazici,YD et al. Concomitant inhibition of epidermal growth factor and vascular endothelial growth factor receptor tyrosine kinases reduces growth and metastasis of human salivary adenoid cystic carcinoma in an orthotopic nude mouse model. Mol Cancer Ther, 2006. 5(11):2696-2705.
23. Burgess,WH and Maciag,T. The heparin-binding (fibroblast) growth factor family of proteins. Annu Rev Biochem, 1989. 58575-606.
24. Batsakis,JG. Tumors of the Nead and Neck: Clinical and Pathological Considerations, 2nd edition. Williams & Wilkins Baltimore 1979.
25. Rossi,G, Cavazza,A, Sturm,N et al. Pulmonary carcinomas with pleomorphic, sarcomatoid, or sarcomatous elements: a clinicopathologic and immunohistochemical study of 75 cases. Am J Surg Pathol, 2003. 27(3):311-324.
26. Matsumoto,T, Fujii,H, Arakawa,A et al. Loss of heterozygosity analysis shows monoclonal evolution with frequent genetic progression and divergence in esophageal carcinosarcoma. Hum Pathol, 2004. 35(3):322-327.
27. Teixeira,MR, Qvist,H, Bohler,PJ et al. Cytogenetic analysis shows that carcinosarcomas of the breast are of monoclonal origin. Genes Chromosomes Cancer, 1998. 22(2):145-151.
28. Adam,P, Zettl,A, Zollner,U et al. Metastasizing vulvar carcinosarcoma with squamous carcinomatous and leiomyosarcomatous differentiation: genetic evidence of clonal origin. Hum Pathol, 2005. 36(10):1143-1147.
29. Fujii,H, Yoshida,M, Gong,ZX et al. Frequent genetic heterogeneity in the clonal evolution of gynecological carcinosarcoma and its influence on phenotypic diversity. Cancer Res, 2000. 60(1):114-120.
30. Gotte,K, Riedel,F, Coy,JF et al. Salivary gland carcinosarcoma: immunohistochemical, molecular genetic and electron microscopic findings. Oral Oncol, 2000. 36(4):360-364.
31. Thompson,L, Chang,B, and Barsky,SH. Monoclonal origins of malignant mixed tumors (carcinosarcomas). Evidence for a divergent histogenesis. Am J Surg Pathol, 1996. 20(3):277-285.
32. Fowler,MH, Fowler,J, Ducatman,B et al. Malignant mixed tumors of the salivary gland: a study of loss of heterozygosity in tumor suppressor genes. Mod Pathol, 2006. 19(3):350-355.
33. Debiec-Rychter,M, Van Valckenborgh,I, Van den Broeck,C et al. Histologic localization of PLAG1 (pleomorphic adenoma gene 1) in pleomorphic adenoma of the salivary gland: cytogenetic evidence of common origin of phenotypically diverse cells. Lab Invest, 2001. 81(9):1289-1297.
34. Tso,CL, Shintaku,P, Chen,J et al. Primary glioblastomas express mesenchymal stem-like properties. Mol Cancer Res, 2006. 4(9):607-619.
35. Jong,K, Marchiori,E, Van der Vaart,A et al. Cross-platform array comparative genomic hybridization meta-analysis separates hematopoietic and mesenchymal from epithelial tumors. Oncogene, 2007. 26(10):1499-1506.
36. Hungermann,D, Roeser,K, Buerger,H et al. Relative paucity of gross genetic alterations in myoepitheliomas and myoepithelial carcinomas of salivary glands. J Pathol, 2002. 198(4):487-494.



Supplemental Figure S1 Hierarchical clustering and heat map of primary epithelial, mesenchymal, and myoepithelial tumors. Cluster I contains mainly neoplasms with mesenchymal features, cluster II contains tumors with few aberrations **(A)** and adenoid cystic carcinomas **(B)**. Cluster III consists of tumors with epithelial origin. Green indicates copy number gains and red deletions. Chromosomes are indicated on the left, alternating in color. From bottom to top – chromosome 1 to chromosome 22.

CHAPTER 8

Summary

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Chapter 8

Summary

Although salivary gland tumors represent only a small portion of all human cancers, they are a major challenge to pathologists and clinicians. Their morphologic diversity, with over 40 different histologic subtypes listed,[1] can frequently impose diagnostic problems. Even in the case of a fairly straightforward diagnosis, prediction of their biologic behavior is difficult.

Since current diagnostic and prognostic parameters, consisting mainly of clinicopathological variables,[2] are not always as reliable as desired, many studies attempted to search for markers of disease on a protein level. Focus of these immunohistochemical investigations comprised mostly of known oncogenes or tumor suppressor genes (such as members of essential cell cycle pathways and cell adhesion factors).[3-5] However, since salivary gland tumors are relatively rare, samples sizes in most of these studies remained small and as a consequence, results were sometimes difficult to interpret. Another limitation of these tumors is the necessity for an extensive follow-up time; recurrences and metastases can develop even after 20 years of diagnosis. Because of the lack of such long time follow-up data, many studies do not possess either reliable or even any prognostic significance.

One cellular component of salivary gland tissue, the myoepithelial cell, has received increasing attention in recent years because of its natural tumor suppressive phenotype.[6] Albeit myoepithelial cells tend to resist transformation, neoplastic myoepithelial cells are a main component of various salivary gland tumors. The complex histologic and biologic characteristics of these myoepithelial cells are also mirrored in the broad morphology and clinical behavior of the tumors they produce. Unfortunately, while it is believed that myoepithelial salivary gland carcinomas, like other neoplastic lesions, develop and progress through an accumulation of alterations in proto-oncogenes and tumor-suppressor genes, genetic events leading to the initiation and progression of these cancers are poorly characterized. Immunoprofiling and genomic analysis of a relatively large group of myoepithelial neoplasms could significantly improve their classification and prognostication.

To this end, the main goal of the research described in this thesis was to evaluate disturbances on a protein and chromosomal level in

salivary gland tumors with myoepithelial differentiation. We aimed to extend our knowledge on (1) the expression of important proteins involved in cell cycle regulation and maintenance of cell identity and (2) the chromosomal aberrations that underlie tumor development.

In **chapter 2**, it is shown in a relatively large group of adenoid cystic carcinomas (ACCs; *n*=18) that this malignancy harbors a large amount of genomic aberrations, in which DNA copy number increases predominate. The most frequent gains include numerous loci of growth factors and their receptors, especially fibroblastic growth factors (FGFs). Notably, gains at loci of growth factors occur more frequently in aggressive ACCs compared to indolent tumors. Survival correlates with the number of genomic aberrations in this neoplasm.

In **chapter 3**, the expression of the proteins $p16^{INK4a}$, E2F1, Cyclin D1, p53 and Ki-67 is determined in 21 paraffin-embedded ACCs by immunohistochemistry. Compared to normal salivary gland tissue, expression levels of all proteins examined are significantly elevated in ACC. In this group of tumors, low Cyclin D1 staining correlates with the occurrence of metastasis during the time of follow-up. Normal salivary gland tissue exhibits a mutually exclusive expression pattern of the two Polycomb group (PcG) complexes. In ACC, this pattern is greatly disturbed, with EZH2 and EED being highly expressed in the presence of BMI-1 and MEL-18. High EZH2 expression is predictive of unfavorable outcome in univariate analysis. Multivariate analysis reveals the presence of recurrence as the most important prognostic factor.

In **chapter 4**, a limited amount of genomic aberrations is described in both benign (*n*=15) and malignant (*n*=12) myoepitheliomas. Frequent DNA copy number changes are detected at loci of growth factors and growth factor receptors (PDGF, EGFR and several FGFs), and common losses are observed at loci of proto-cadherins. Unsupervised clustering reveals separate clusters with a clear correlation to malignancy.

In **chapter 5**, protein expression levels of the cell cycle regulators $p16^{INK4a}$, p53, Ki-67, E2F1, and Cyclin D1 is investigated in a large group of benign (*n*=49) and malignant (*n*=30) paraffin-embedded

myoepitheliomas by immunohistochemistry. In benign tumors, overexpression of Rb-p16 pathway members is present. In addition to this deregulation, malignant myoepitheliomas show overexpression of p53. The five recurrences of histologically benign tumors in this group show similar immunoprofiles as the carcinomas by demonstrating deregulation in both pathways. As in normal salivary gland tissue, a mutually exclusive expression pattern of the two PcG group complexes is observed in the benign tumors while malignant myoepitheliomas overexpress EZH2.

Finally in **chapter 6**, the genomic profile of a third type salivary gland tumor with myoepithelial differentiation is investigated, namely a carcinosarcoma. In this study, a high-resolution oligonucleotide array is used to examine the genomic profiles of the two distinct components in the tumor separately. Both elements show a high number of alterations, which are shared for 75%. The extensive overlap between the two profiles indicates a monoclonal origin for the two components.

Samenvatting

Hoewel speekselkliertumoren vrij zeldzaam zijn, vormen zij een grote uitdaging voor pathologen en clinici. De classificatie, bestaande uit meer dan 40 verschillende soorten,[1] berust volledig op histopathologische kenmerken en zorgt regelmatig voor diagnostische problemen. En zelfs in het geval van een eenduidige diagnose, is het voorspellen van het biologisch gedrag van de tumor moeilijk.

Omdat de huidige diagnostische en prognostische parameters, bestaande uit voornamelijk clinicopathologische variabelen,[2] niet voldoende betrouwbaar zijn, hebben talrijke onderzoeken getracht voorspellende markers op eiwitniveau te vinden. De focus van deze studies lag voornamelijk op bekende oncogenen en tumor-suppressor genen (zoals essentiële celcyclus en celadhesie eiwitten).[3-5] Echter, omdat speekselkliertumoren relatief weinig voorkomen, bleef het aantal onderzochte tumoren beperkt en waren de resultaten daardoor soms moeilijk te interpreteren. Een andere beperking van deze tumoren is de noodzaak van langdurige controle; locoregionale recidieven en uitzaaiingen op afstand kunnen zelfs 20 jaar na behandeling van de primaire tumor optreden. Derhalve is langdurige follow-up van patiënten nodig om een uitspraak te kunnen doen over de prognostische waarde van een onderzochte marker.

Een onderdeel van het speekselklierweefsel, de myoepitheliale cel, is wegens zijn tumor-suppressieve fenotype steeds meer in de aandacht gekomen de laatste jaren.[6] Ondanks dat myoepitheliale cellen redelijk resistent zijn tegen maligne transformatie, zijn neoplastische myoepitheliale cellen een belangrijk onderdeel van diverse typen speekselkliertumoren.

Ondanks de veronderstelling dat myoepitheliale speekselkliertumoren, zoals andere neoplasmata, het resultaat zijn van een accumulatie van verstoringen in proto-oncogenen en tumor-suppressor genen, zijn de genetische afwijkingen die tot het ontstaan van deze kankers leiden nog slecht gekarakteriseerd. Immunoprofilering en genomische analyse van een relatief grote groep myoepitheliale tumoren zou de inzichten hierin significant kunnen verbeteren.

Derhalve heeft het onderzoek dat beschreven is in dit proefschrift de afwijkingen op eiwit en chromosomaal niveau in speekselkliertumoren

met myoepitheliale differentiatie geëvalueerd. We wilden onze kennis uitbreiden wat betreft (1) de expressie van essentiële eiwitten die betrokken zijn bij de celcyclus en het behoud van de celidentiteit en (2) de chromosomale aberraties die ten grondslag liggen aan het ontstaan van de tumor.

In **hoofdstuk 2** wordt een relatief grote groep adenoïd cystische carcinomen (ACC; *n*=18) onderzocht, waarbij blijkt dat deze maligniteit een grote hoeveelheid genomische afwijkingen vertoont met een voorkeur voor toename ("gains") in delen van het genetisch materiaal. De meest frequente DNA gains bevatten loci van vele groeifactoren en groeifactorreceptoren, voornamelijk van fibroblastische groeifactoren (FGFs). Het is opmerkelijk, dat agressieve ACCs vaker DNA toename op de loci van groeifactoren vertonen dan tumoren met een meer indolent gedrag. Het blijkt ook dat overleving van patiënten met ACC afhankelijk is van het aantal genomische afwijkingen in de tumor.

In **hoofdstuk 3** wordt de expressie onderzocht van de eiwitten $p16^{INK4a}$, E2F1, Cycline D1, p53 en Ki-67 in 21 paraffine-ingebedde ACCs met behulp van immunohistochemie. Hierbij wordt aangetoond, dat de expressie van alle onderzochte eiwitten in ACC verhoogd is in vergelijking met normaal speekselklierweefsel. In de onderzochte groep correleert geringe Cycline D1 aankleuring met het ontstaan van metastases tijdens follow-up. Expressie van de twee complexen van de Polycomb groep eiwitten (PcG), die in normaal speekselklierweefsel afzonderlijk van elkaar voorkomen, is in ACC verstoord, aangezien EZH2 en EED in de tumorcellen tegelijk met BMI-1 en MEL-18 voorkomen. Hoge EZH2 expressie voorspelt een slechte prognose in univariate analyse. Multivariate analyse geeft het ontstaan van recidieven als meest belangrijke prognostische factor.

In **hoofdstuk 4** wordt beschreven dat zowel goedaardige (*n*=15) als kwaadaardige (*n*=12) myoepitheliomen een beperkt aantal genomische afwijkingen hebben. Veelvuldig voorkomende DNA gains worden gedetecteerd op de loci van groeifactoren en groeifactorreceptoren (PDGF, EGFR en verschillende FGFs) en frequente deleties worden waargenomen op de loci van protocadherines. Cluster analyse geeft

afzonderlijke clusters waarbij er een duidelijke correlatie is met maligniteit.

In **hoofdstuk 5** wordt in een grote groep goedaardige (*n*=49) en kwaadaardige (*n*=30) paraffine-ingebedde myoepitheliomen de expressie beschreven van de celcyclus regulatoren $p16^{INK4a}$, p53, Ki-67, E2F1 en Cyclin D1. Leden van de Rb-p16 “pathway” komen verhoogd tot expressie in goedaardige tumoren. Naast deze deregulatie hebben de maligne myoepitheliomen ook overexpressie van p53. De vijf recidieven van histologisch goedaardige tumoren in de onderzochte groep hebben vergelijkbare eiwitprofielen met de carcinomen, waarbij beide “pathways” gedereguleerd zijn. In goedaardige tumoren komen, net als in normaal speekselklierweefsel, de twee PcG complexen afzonderlijk van elkaar tot expressie, terwijl in kwaadaardige myoepitheliomen EZH2 verhoogd tot expressie komt.

Tenslotte wordt in **hoofdstuk 6** het genomische profiel beschreven van een derde type speekselkliertumor met myoepitheliale differentiatie, namelijk een carcinosarcoom. In deze studie werd een hoge-resolutie oligonucleotide array gebruikt om de genomische profielen van de twee verschillende tumorcomponenten afzonderlijk van elkaar te bekijken. Beide elementen laten een groot aantal afwijkingen zien, die voor 75% overlappen. Deze uitgebreide homologie tussen de twee profielen duidt op een monoklonale oorsprong van de twee componenten.

Chapter 8

References

1. Barnes,L, Eveson,JW, Reichart,P et al. World Health Organization Classification of Tumours: Head and Neck Tumours. IARC*Press* Lyon 2005.
2. Terhaard,CH, Lubsen,H, Van der Tweel,I et al. Salivary gland carcinoma: independent prognostic factors for locoregional control, distant metastases, and overall survival: results of the Dutch head and neck oncology cooperative group. Head Neck, 2004. 26(8):681-692.
3. Franchi,A, Gallo,O, Bocciolini,C et al. Reduced E-cadherin expression correlates with unfavorable prognosis in adenoid cystic carcinoma of salivary glands of the oral cavity. Am J Clin Pathol, 1999. 111(1):43-50.
4. Yamamoto,Y, Wistuba,II, Kishimoto,Y et al. DNA analysis at p53 locus in adenoid cystic carcinoma: comparison of molecular study and p53 immunostaining. Pathol Int, 1998. 48(4):273-280.
5. Shintani,S, Mihara,M, Nakahara,Y et al. Infrequent alternations of RB pathway (Rb-p16INK4A-cyclinD1) in adenoid cystic carcinoma of salivary glands. Anticancer Res, 2000. 20(3B):2169-2175.
6. Sternlicht,MD, Kedeshian,P, Shao,ZM et al. The human myoepithelial cell is a natural tumor suppressor. Clin Cancer Res, 1997. 3(11):1949-1958.

Curriculum Vitae

De auteur van dit proefschrift werd op 26 november 1976 geboren te Kecskemét in Hongarije. Nadat ze op haar elfde was verhuisd naar Nederland, behaalde ze in 1997 haar Gymnasium diploma aan de OSG Bataafse Kamp te Hengelo, Overijssel. In hetzelfde jaar begon ze aan de studie Medische Biologie aan de Vrije Universiteit te Amsterdam. Tijdens deze studie verrichtte zij onderzoek bij de afdeling Pathologie van de Vrije Universiteit, waar ze onder begeleiding van Dr. FM. Raaphorst de klonale samenstelling van T cellen in maag en cervixtumoren onderzocht. Haar tweede wetenschappelijke stage heeft ze gelopen bij de afdeling “Microbiology and Immunology” aan de Vanderbilt University Medical Center in Nashville, Tennessee, VS (Prof.dr. E. Oltz). Gedurende vijf maanden werkte ze aan de epigenetische en biochemische mechanismen die betrokken zijn bij de assemblatie van de T cel receptor. Na haar afstuderen in maart 2004 is zij in dienst getreden als assistent in opleiding (AIO) bij de afdeling Orale Pathologie van het Academische Centrum Tandheelkunde Amsterdam (ACTA), onder begeleiding van Prof.dr. E. Bloemena en Prof.dr. I. van der Waal. Het onderzoek dat zij hier heeft uitgevoerd heeft geresulteerd in dit proefschrift. Sinds juni 2008 is zij werkzaam als postdoc op de afdeling Moleculaire Pathologie van het Erasmus Medisch Centrum, te Rotterdam. In de groep van Prof.dr. E. Zwarthoff werkt zij aan de functionele analyse van het MN1 oncogen in acute myeloide leukemie.

Chapter 8

Dankwoord

Zo.Het.Is.Af.
En bij het afsluiten van dit boekje begin ik aan een hele lijst van collega's, vrienden en familieleden die in meer of mindere mate hebben bijgedragen aan de totstandkoming van dit proefschrift. Natuurlijk was het makkelijker geweest om in koeienletters "IEDEREEN BEDANKT!" op te schrijven, maar dan zou ik toch echt veel mensen te kort doen.

In de eerste plaats mijn promotoren, Professor Bloemena en Professor Van der Waal. Beste Elisabeth, hoe had ik na zo een korte kennismaking kunnen denken, dat ik zo op mijn plaats zou zijn bij jou? Zelden kwam het voor, dat ik de gang in kwam lopen en je deur stond niet letterlijk en figuurlijk voor me open. Jouw gezellige persoonlijkheid en kritische en nuchtere kijk op (wetenschappelijke) zaken hebben ervoor gezorgd, dat ik met veel plezier terugkijk op de afgelopen vier jaar. Je bent een ware inspiratie, als wetenschapper en als persoon! Bedankt voor ALLES!
Prof. Van der Waal, ik wil u hartelijk bedanken voor een hele prettige samenwerking. Ik heb veel geleerd van uw kritische commentaar op de stukken en heb uw interesse, op wetenschappelijk en persoonlijk vlak, erg gewaardeerd.
De leden van de lees- en promotiecommissie wil ik bedanken voor hun tijd en aandacht die ze hebben besteed aan het lezen van het proefschrift en aan het voeren van de oppositie. Frank, you and I go way back. Als groen studentje heb je me al onder je vleugels genomen. Je hebt op me in zitten praten om naar de VS te gaan voor mijn stage en vervolgens heb je me weten te overtuigen om als AIO aan het werk te gaan. Kortom, de leukste periodes in mijn korte loopbaan heb ik te danken aan je koppigheid. En daar ben ik je enorm dankbaar voor!

De volgende groep collega's die ik natuurlijk wil bedanken zijn de mensen die de klappen van het AIO-zweepje ook kennen: Sas Cil, Sas Santegoeds, Rieneke, Annelies, Joost, Johan, Maria, Maikel, Reneé, Wouter, Inge, Floor, Jelle, Annemieke, Bart, Pepijn, Murat, Dorien, Kirsten, Paul, Berbel en Jillian bedankt voor de gezellige uurtjes, de borrels, maar ook voor de serieuze pep-talks.

Veel tijd (tijdens werkbesprekingen en de vrijdagmiddagborrels) heb ik ook doorgebracht met de TP-ers. Niet alleen hebben Gerrit, Beatriz, Tineke, Marianne, Linda, Rinus, Cindy, en Begoña mij altijd met raad en daad terzijde gestaan met al mijn CGH problemen (en dat waren er veel!), maar het was ook altijd gezellig om even binnen te stappen met een kopje thee.

Van de vrouwengroep kom ik automatisch bij de mannen: de Microarray groep. Bauke, Paul, Serge, François, Alan, Mark, Wessel, Sjoerd en Thijs wil ik bedanken voor hun adviezen en hulp en de nooit-meer-te-vergeten feestjes!

Bij de afdeling Pathologie krijgen de medewerkers van de diagnostiek en Immuno een speciaal bedankje voor hun hulp met alle kleuringen: Wim Vos, Thea, Mohammed, Wil, TR, Helma en Angelique. Beste Wim, je krijgt honderden bedankjes van me voor de evenzoveel coupes die je voor me gesneden hebt!

Regelmatig heb ik de fotografen van de PA gestoord tijdens hun theepauze met (spoed)klusjes over mijn figuren. Jaap en Ron, ik ben jullie heel veel dank verschuldigd voor jullie hulp, uitleg en gezellige gesprekjes. Zonder jullie was mijn drukwerk lang niet zo mooi geworden!

Special thanks for the members of Oral Cell Biology: Marlene, Ana, Aviral, and Agnes. I always had a great time with you guys during our annual Lunteren meeting and also during drinks after working hours. Thanks for everything and I am looking forward to stay in touch!

Van de oude, naar de nieuwe groep. Beste Magda, mijn moleculaire-vraagbaak en voorbeeld-moeder, bedankt voor ál je hulp tijdens de (soms hobbelige) overgang van translationeel naar moleculair onderzoek (“Hij doet het niet! Misschien handig om hem aan te zetten!?!”). Ik heb nú al zoveel van je geleerd en deels daarom, ook al kijk ik erg uit naar jouw promotie, hoop ik dat we nog lang zij en zij mogen werken. De rest van de Zwarthoff-groep, Ellen, Tahlita, Kirstin, Martijn, Samira, Lucie, Raju, Angela, Irene en Marcel wil ik natuurlijk ook bedanken voor hun hulp, wetenschappelijke input en broodnodige wijn- en haaradviezen.

Mijn lieve vriendinnetjes, Mieke, Clari, Cindy, Jess en Sas. Na periodes van ploeteren kon ik altijd bij jullie terecht voor de nodige ontspanning

tijdens een heerlijke maal en een goed glas bier. Wat heb ik met jullie veel afgelachen...Dank jullie wel voor onze gesprekken over pietluttige én belangrijke dingen! Jess en Sas, fantastisch dat jullie mijn paranymfen wilden zijn. Lieve Sas, mijn derde hersenhelft, zonder jouw hulp tijdens de soms gekmakende analyses (“ongelooflijk!!”), had ik het echt niet gered. Lieve Jess, het was altijd heerlijk toeven op je balkonnetje waar we een scala aan onderwerpen hebben besproken. Je bent een super vriendin en ik hoop, dat nog vele vrijdagmiddagen zullen volgen!

Dear Roshi, we became friends in Nashville and we have been close ever since. Thanks for your friendship and I am looking forward to many more visits!

Noël en Marlies, Sjarkie, Suus, Mark en Liselot, Patrick, Geppie, Richard, Bas, Linda en Joost, Teun en Fleur, Dirk en Faezeh, Luitzen en Nicole en de vele anderen die ik vast ben vergeten, bedankt voor de gezelligheid!

Mijn mini-familie hier in Nederland, anya, Harry, Aliz, Jonas, Jacqueline en Gerwin, bedankt voor jullie interesse, geduld en onvoorwaardelijke steun. Jullie waren een onvervangbaar vangnet en zonder jullie was het echt niet gelukt. Harry, bedankt voor al die keren dat je me minstens een half uur liet kletsen over het wel-en-wee van een AIO. Anya en Aliz, samen zijn we de drie musketiers, lief en leef gedeeld, bedankt dat jullie me zo gesteund hebben! A magyar családot, Vékony mamát, Bárnai mamát és papát, keresztanyut és keresztaput, Ricsit és Lindát szeretném megköszönni hogy mindig szeretettel gondoskodtak rólam. Soha nem fogom elfelejteni a kellemes időket amiket eggyütt töltöttünk.

Mijn “andere” familie, Riet, Ton, Ingeborg, Joost, Nienke en Thijs. De weekenden in Roosendaal waren altijd een heerlijk rustpunt. Riet, je telefoontjes met de vraag “Kan ik wat voor je doen?” waren soms net dat extra steuntje in de rug dat ik nodig had. Dank jullie wel voor de goede zorgen, interesse en de vele potjes muurtje-trap!

Lieve Martijn, jouw nuchtere en rustige Brabantse karakter was precies wat mijn Hongaarse persoontje nodig had in deze laatste maanden van stress. Ondanks dat je ogen soms wat glazig werden als ik over mijn werk begon, kon ik altijd bij je terecht. Je kunt me laten lachen als geen ander en voor dat, en nog duizenden andere dingen, dank je wel.